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Volume 47, Supplement 1, May 1956

SYMPOSIUM

ON

STRUCTURE OF ENZYMES AND PROTEINS

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PHILADELPHIA
1956

SYMPOSIUM

ON

STRUCTURE OF ENZYMES AND PROTEINS

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INTRODUCTION

The interrelations of enzymes and protein structure are of fundamental importance to an understanding of the functions of living cells and basic to the study of the effects of radiation on living organisms and modification of their radiation response. For this reason, it seemed advisable to make the subject of the Eighth Annual Biology Research Conference "Enzyme and Protein Structure." An evening was devoted to a discussion of the developments in the study of the structure of deoxyribonucleic acid and its relation to proteins. The papers and a good part of the discussion are reproduced in this volume.

This meeting, like the previous ones, was sponsored by the Biology Division of the Oak Ridge National Laboratory and the Division of Biology and Medicine of the United States Atomic Energy Commission. One innovation was introduced for this conference: instead of holding it at Oak Ridge, it was convened at the Mountain View Hotel at Gatlinburg, Tennessee at the entrance of the Great Smoky Mountains National Park, about 65 miles from Oak Ridge. This proved to be a highly successful arrangement, which may set a pattern for further meetings.

Arrangement of this program was done largely through the efforts of Dr. David G. Doherty. Grateful acknowledgment is accorded to Dr. Doherty and to Dr. Raymond Shapiro for their valuable advice during the preparation of this symposium for publication.

Previous symposia in this series are:

1948 — Radiation Genetics
1949 — Radiation Microbiology and Biochemistry
1950 — Biochemistry of Nucleic Acids
1951 — Physiological Effects of Radiation at the Cellular Level
1952 — Some Aspects of Microbial Metabolism
1953 — Effects of Radiation and Other Deleterious Agents on Embryonic Development
1954 — Genetic Recombination

Alexander Hollaender

THE NATURE OF THE PROTEIN MOLECULE: PROBLEMS OF PROTEIN STRUCTURE[1]

FELIX HAUROWITZ
Department of Chemistry, Indiana University, Bloomington

TWO FIGURES

In any discussion of protein structure, different types of bonds must be considered. They may be classified as two groups: (1) covalent bonds between amino acids, responsible for their sequence and also for branching of the peptide chain, and (2) bonds due to electrostatic interaction between polar groups (e.g., hydrogen bond formation) and to van der Waals forces between nonpolar groups. Lumry and Eyring ('54) have proposed to classify three types of structure: primary structure formed by covalent bonds, secondary structure maintained by hydrogen bonds, and tertiary structure caused by van der Waals forces. They consider changes of the secondary structure as irreversible and those of the tertiary structure as reversible. However, changes in the secondary structure in most cases involve also changes in the tertiary structure; therefore, it is difficult to differentiate between these two types of structures.

In this paper, the first discussion will be on problems of the chemical constitution, i.e., of the primary structure formed by covalent bonds. In a second section, the fine structure of the protein molecules will be treated, i.e., the folding or coiling of their peptide chains, and reactions involving changes in this structure. The last section will concern the problem of heterogeneity of the proteins.

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[1] The experimental work described in this paper was supported by contracts of Indiana University with the U. S. Atomic Energy Commission and the Office of Naval Research, and by research grants from the U. S. Public Health Service and the American Cancer Society.

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THE PROBLEM OF BRANCHED PEPTIDE CHAINS

The primary structure of proteins is determined by the number and sequence of amino acids and the mode of branching of the peptide chains. This structure has been completely elucidated in some of the smaller protein or peptide molecules such as insulin (Sanger and Thompson, '53), ACTH (Bell, '54), and the posterior pituitary hormones (du Vigneaud *et al.*, '53; Tuppy, '53; Acher and Chauvet, '53). It seems that careful analysis of partial hydrolyzates will also lead to the complete description of the structure of larger molecules. One of the main problems is whether the molecules of the typical proteins consist of a single, straight peptide chain or a branched chain, or whether they are formed by a number of parallel chains held together by covalent dithio bonds, as in insulin, or by electrostatic and/or van der Waals forces.

In this laboratory, examination has been made of the problem of SS bonds between parallel peptide chains and that of chain branching (Haurowitz and Kennedy, '52). Three of the possible structures are shown in figure 1. In order to discover SS bonds between parallel peptide chains (fig. 1b), determinations were made of the light scattering of proteins dissolved in thioglycolate at *p*H 8.5, and in control experiments in glycolate of the same ionic strength and *p*H. Dithio bonds were expected to be split by thioglycolate and the molecular weight, determined by light scattering, should therefore decrease. In none of the four examined proteins was a decrease of the molecular weight in thioglycolate observed, nor was free cysteine split off by an excess of thioglycolate. The conclusion must be, therefore, that the examined proteins consist of a single straight or branched peptide chain (fig. 1a) and not of a number of chains held together by dithio bonds (fig. 1b) nor of a peptide chain with cystine molecules bound only by one-half of their molecule (fig. 1c).

In another series of experiments, investigations were made of the reaction taking place when proteins are treated with ammonium thiocyanate in acetic anhydride. Thiocyanate reacts under these conditions with the C-terminal groups forming thiohydantoin rings. When radioactive thiocyanate containing S^{35} or C^{14} was used for these experiments, incorporation of a large number of thiocyanate residues was found in all the typical high-molecular proteins (see table 1) (Haurowitz *et al.*, '54). Since the radioactivity was not washed out by equilibration with nonradioactive thiocyanate, it cannot be caused by the binding of thiocyanate ions. Nor can the incorporation of thiocyanate be caused by cleavage of the protein during the treatment with acetic anhydride; for no unusual incorporation was observed with small molecules such as insulin or salmine. Moreover, the reaction products of proteins were insoluble, whereas those obtained by condensing thiocyanate with insulin or other smaller molecules were soluble in water. As shown by reaction (1), thiohydantoins are formed by only those carboxyl groups that are adjacent to an α-amino or -imino group. It is generally assumed that such groups are present at only the C-terminus of the peptide

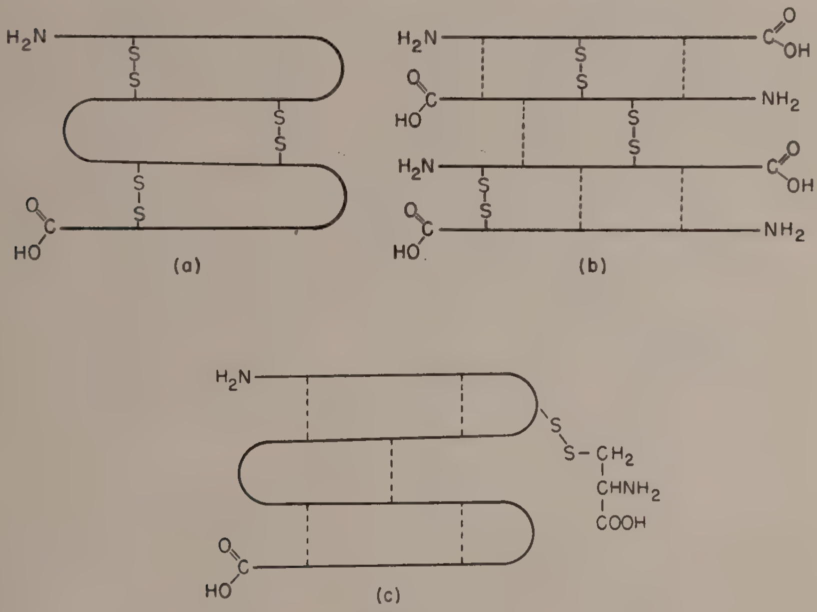


Figure 1

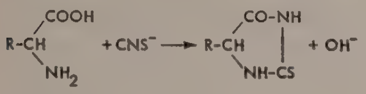
TABLE 1

Moles of thiocyanate incorporated per mole of protein

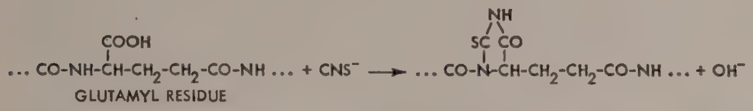
PROTEIN	MOLECULAR WEIGHT [a]	C^{14}	S^{35}	PROTEIN	MOLECULAR WEIGHT [a]	C^{14}	S^{35}
Ovalbumin	44,000	7.8	6.6	Ribonuclease	13,000	1.4	. .
Serum albumin (beef)	68,000	8.3	10.0	Tyrocidin	1,300	0.2	. .
γ-Globulin (beef)	165,000	39.4	19.0	Pepsin	35,000	4.7	. .
Globin (beef)	66,000	5.0	7.3	Collagen	. . . . .	4.4	4.4
Edestin	310,000	65.0	50.0	Gelatin	. . . . .	9.7	. .
Casein	. . . . . .	12.6	12.7	α-Fibroin	56,000	3.3	. .
Gliadin	. . . . . .	13.7	. . .	Insulin	12,000	2.6	. .
Chymotrypsin	22,000	2.2	. . .	Salmine	8,000	0.16	. .
Lysozyme	17,000	2.3	. . .	Bacitracine	2,000	0.11	. .

[a] If the molecular weight is not known, the number of incorporated moles per 10^5 g of substance is recorded.

chains. However, a few years ago it was pointed out that γ-bound glutamyl residues might exist in the proteins (Haurowitz and Bursa, '49). Such residues would combine with thiocyanate to form thiohydantoin rings [eq. (2)].



(1)



(2)

Although these experiments do not prove directly the presence of γ-glutamyl residues, they give some evidence for their presence in proteins. Other evidence has been presented by Fromageot (Jollès and Fromageot, '51) who, after reduction of proteins with $LiAlH_4$ and hydrolysis of the reduction product, found small amounts of 4-amino-5-hydroxyvaleric acid. γ-Glutamyl residues occur in nature in glutamine as well as glutathione, in folic acid, and in the polyglutamic acid from anthrax bacilli. It would not be surprising, therefore, to find such groups in genuine protein molecules.

THE SURFACE OF GLOBULAR PROTEIN MOLECULES

In the secondary structures, the most important problem seems to be the spatial distribution of the amino acids in the globular protein molecules. Although chromatography has allowed quantitative determination of all the amino acids of about sixty or more proteins, this gives no insight into the problem of the spatial distribution of the amino acids in the protein molecule. If a protein model consisting of a long peptide chain is built up, it can be folded in an infinite number of fashions; for instance, in such a manner that all cationic groups are in the surface and all anionic groups in the interior (fig. 2a). The reverse situation with all anionic groups in the surface is shown in figure 2b. A model can also be produced which has both positive and negative groups in the surface or nonpolar groups only in its surface (fig. 2c, d). Obviously, the properties of the four molecules (2a–d) will be very different, although their amino acid composition may be identical. It can be reasonably assumed that the biological activities of proteins depend on only the outermost shell. The groups in the interior are inaccessible to large counter molecules. Therefore, the specific interaction of enzymes with substrates or of antigens with antibodies will depend on the peripheral groups only. These groups will be called ''exo groups'' to differentiate them from the ''endo groups,'' which are buried in the core of the globular protein molecules and are, therefore, inaccessible to macromolecular substrates or other reactants.

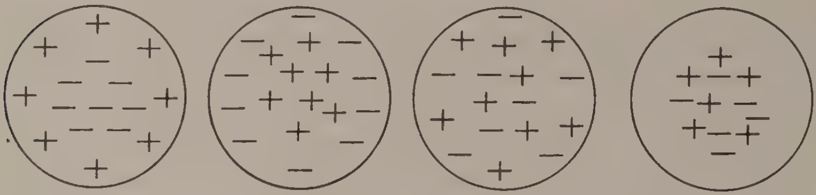

(a) (b) (c) (d)

Figure 2

It may seem unusual to speak of the shell, or periphery, and the interior, or core, of a molecule when this molecule consists of a straight or slightly branched peptide chain. Indeed, the terms ''periphery'' and ''core'' cannot be applied to nonpolar macromolecules, such as rubber. If nonpolar molecules are dissolved in organic solvents, they unfold and on drying, refold randomly. However, when proteins are dissolved and recrystallized or when they are salted out and redissolved in water, their specificity is unchanged. This proves convincingly that the protein molecule survives these processes without alterations of its original three-dimensional structure. Hence there is justification for differentiating exo from endo groups or, in other words, for discussing the topography of protein molecules, i.e., the distribution of their reactive groups in space.

In changing the folding or coiling of the peptide chain of a protein molecule, the chemical constitution is not altered, since it is determined by the number and sequence of the amino acids. Nor is the optical configuration altered. A new term had to be coined for changes of this type; Kuhn ('36) designates the definite arrangement of the peptide chain as "constellation," whereas Lumry and Eyring ('54) use the term "conformation." Unfortunately, very few methods are available for investigating the constellation or conformation of proteins.

Some information of the exo groups of proteins can be gained by use of large counterions, particularly dyes. Thus investigations of Klotz ('50), Karush ('50), and Haurowitz *et al.* ('52) show that serum albumins combine readily with dye anions whereas serum globulins do not combine. Since the ratio of basic to acidic groups is higher for globulins than for albumins, the combination of the dye anions with serum albumin cannot be explained by the presence of randomly distributed basic groups in the proteins. Attempts have been made to correlate the affinity of serum albumin for acidic dyes with the number of hydroxyl groups in the molecule (Klotz, '50) or with the adaptability of the molecular surface (Karush, '50). Steric factors may, indeed, play an important role. However, the simplest explanation for the combination of serum albumin with large dye anions would be the presence of a number of basic exo groups (fig. 2a). It must be admitted that there is no other proof for such a distribution of the charges. It may be mentioned that most of the enzymes seem to behave like serum globulins in that they fail to combine with anionic dyes. If our assumption is right, it would mean that the enzyme molecules are free of or poor in basic exo groups.

Efforts have been made to get analogous information on the presence of *acidic exo groups,* which are able to react with the large basic salmine ions (Haurowitz, M. Kennedy, and J. Turner, unpublished experiments, '54). Serum albumin in 1% solution gave no precipitate when mixed at *p*H 7–9 with an equal volume of 1% salmine in the presence of 1% NaCl;

serum globulin gave a weak precipitate under these conditions. In contrast to the native proteins, the denatured proteins, albumins as well as globulins, gave intense precipitation with salmine over the whole pH range from 6 to 10. This seems to indicate that the native proteins have no acidic exo groups and that these groups become accessible after denaturation only.

In order to get some information about *nonpolar exo groups,* various proteins were injected into the interface between aqueous buffer solutions and p-xylene or bromobenzene. The interfacial pressure of such protein films is equal to the difference between the interfacial tensions of the protein solution and the protein-free buffer solution. The film pressure rose from very low values to approximately 15 dynes/cm for most of the investigated proteins. However, globin, the protein moiety of hemoglobin, had an interfacial pressure of approximately 25 dynes/cm. On addition of hemin, this pressure decreased to the much lower value (15 dynes/cm) of hemoglobin (Haurowitz *et al.,* '53). It is assumed that the high interfacial pressure of globin is caused by the presence of numerous nonpolar groups and that heme combines with these groups, in this manner lowering the affinity of globin for the oil phase, and its high interfacial pressure (Haurowitz *et al.,* '53).

In some cases, information on the exo groups may be obtained by immunochemical methods. Before evaluating results obtained by immunochemical research, the question may be raised as to how the modern views on a helical structure of the peptide chains can be reconciled with the idea of an inaccessible protein core different from the biologically active surface. At first sight, there seems to be no correlation between these two aspects — one of them based on biological properties, the other on a regularity of structure revealed by X-ray investigations. Difficulties would arise, however, if rigid helices of strictly parallel axes are postulated for all proteins. Such an idea can hardly be reconciled with the complementariness of antibodies, with their spatial adaptation to the determinant groups of the antigens. Obviously, this adaptation involves slight deviations from the normal structure and is in contradiction to an ''ideal'' helical structure with rigid parallel helices only. The single peptide chain structure of many, if not all, proteins implies folding of the helices at their ends, i.e., at the surface of the protein molecules. Accordingly, the surface of the protein molecules, which is responsible for its high specificity and its biological activity, must be particularly rich in loops of the helices and relatively poor in parallel rigid helices.

Although the helical model is still the most satisfactory model for many proteins, it must be emphasized that it is no more than an oversimplified model, which may undergo more or less drastic changes in future.

One of the interesting consequences of the helical model is that it reduces enormously the number of possible conformations, and thereby, the number of possible amino acid arrangements in the surface of the protein molecule. From immunochemical data, it can be concluded that the surface ''patch'' of an antigen molecule that combines with one antibody molecule is not larger than approximately 100–200 A^2, corresponding to an area occupied by 3–4 amino acid residues. If sixteen different types of amino acid residues are assumed, it is found that there are 3360 possible combinations of three and 43,680 combinations of four amino acids. This would then be the number of possible specific patches at the surface of all protein molecules. According to this view, there should not exist billions nor even millions of different antibodies, but probably less than 50,000 types. This number may well satisfy the postulates of serologists; for cross reactions of antibodies with heterologous antigens are known and are attributed to the direction of the specificity of the antibodies against small chemical groups, not against the antigen molecule as an entity. For instance, in an animal injected with iodinated arsanilazo ovalbumin, antibodies combining with the diiodotyrosine group, other antibodies combining with the azophenylarsonic acid group, and a third type of antibodies combining with the unknown determinant groups of ovalbumin are produced. The three types of antibodies can be separated from one another by suitable test antigens (Haurowitz and Schwerin, '43). Natural antigens, likewise, may give rise to the formation of identical antibodies. Thus the antibody against blood group substance A combines not only with this substance, but also with the capsular antigen of pneumococcus type XIV (Kabat *et al.*, '48). Heidelberger and his coworkers have made use of such antibodies for the indentification of definite oligosaccharide residues in polysaccharides (Heidelberger *et al.*, '54). Unfortunately, the antigenic groups of the genuine proteins are not yet known and, therefore, an analogous use cannot be made of antibodies as reagents for the presence of definite arrangements of amino acids in the periphery of globular protein antigens. But this approach should certainly be kept in mind.

HETEROGENEITY OF THE PROTEINS

The discussion of antigen-antibody relations leads to the last problem to be discussed here — the problem of heterogeneity of proteins. The interrelation of antigens and antibodies is attributed to mutual complementariness of their shape. They can, thereby, approach each other so closely that the short-range van der Waals forces become efficient, and attraction results. Since the structure of the injected antigen is given, formation of a complementary structure in antibody molecules must be caused by a kind of adjustment. Two different mechanisms by which complementariness may be accomplished have been taken into consideration: (1) folding of the peptide chains during their formation from amino acids, and (2) rearrangement of the peptide chains of a protein by unfolding and refolding. The first of these hypotheses was proposed by Breinl and Haurowitz ('30), Mudd ('32), and Alexander ('31), whereas Pauling and Campbell ('42) claimed the manufacture of antibodies *in vitro* by incubating normal serum globulins with antigens under conditions of reversible denaturation. The formation of antibodies *in vitro* was, however, not confirmed (Haurowitz *et al.,* '46; Morrison, '53). Most probably the complementariness of the antibody structure is accomplished as a result of folding of the peptide chains during their synthesis from amino acids.

Some of the antibodies differ from normal serum globulins by their molecular weight or by other slight changes in the physical chemical properties. However, most of the antibodies have the same composition and the same properties as normal serum globulins. In this laboratory, antibodies have been produced against a strongly basic antigen containing quaternary ammonium groups as determinant groups, and other antibodies against acidic antigens. The antibodies were precipitated by the homologous antigens, dissociated from the precipitates, and investigated for their amino acid composition. Within the error limits of the analytical methods, the composition of the two antibodies was the same (F. Haurowitz, R. L. Hardin, M. Zimmerman, and J. Horowitz, unpublished experiments, '54). It is, therefore, believed that antibodies differ from each other and from normal globulins essentially by their conformation, i.e., by differences in the mode of folding of their peptide chains. Since antibodies are formed by a kind of adaptation, formation of well and poorly adapted antibodies can be imagined. Indeed, there is strong evidence for the presence of antibodies with different degrees of affinity for the antigen molecules. The immune serum probably contains a complete spectrum of intermediates between normal globulins and antibodies.

The question may be raised, therefore, whether the normal blood serum also contains a series of heterogeneous γ-globulins. And it may be questioned further whether any of the so-called pure proteins, isolated from animal or vegetable sources, are homogeneous (Haurowitz, '50). Colvin *et al.* ('54), in a review article, came to the conclusion that all the proteins, with two possible exceptions, are heterogeneous when investigated by chemical, physicochemical, and immunological methods. In evaluating this result, it must be kept in mind that complete homogeneity can never be proved and that the proof for heterogencity depends on the availability of analytical methods suitable for differentiation. It is very probable, therefore, that all proteins are populations of individual molecules, differing from each other by their conformation, i.e., the distribution of the amino acid residues in the three-dimensional space occupied by the molecule, differing also in the distribution of their charges, their biological activity, and possibly in other properties.

Colvin *et al.* ('54), after careful and critical analysis, come to the conclusion that the heterogeneity of protein preparations is not caused by contamination by other molecules or by artifacts produced by the methods of preparation, but that it is a normal phenomenon caused by inherent variations. These variations do not seem to consist of discrete steps but rather of continuous changes, resulting in a family of molecules related to one another like the members of a culture of cells raised from a single cell.

All this may be bewildering and embarassing for the classical chemist who uses the terms "pure substance" and "crystalline protein" for ideal systems consisting of identical molecules. It may be very welcome, however, to the biologist because such a concept bridges the gap between protein molecules and animated or semianimated units such as the viruses, the cytoplasmic granules, or the nuclear genes. Biologists, when speaking of a "species" or a "pure strain," do not mean a system of identical individuals, but a family of very similar individuals. It would seem that, in protein chemistry, a switch must be made from the classical chemical to a truly biological-chemical point of view; apparently, "protein species" or "protein families" are being dealt with rather than identical protein molecules.

It may be asked finally how the variations of inanimate protein molecules can be explained. This question is closely connected with the problem of the biosynthesis of proteins. This aspect of the problem will not be discussed here, since nothing new can be contributed to previous speculations of the author on the mechanism of protein formation or replication

(Haurowitz, '50) or to similar speculations of others (Dounce, '52; Lipmann, '54). It ought to be kept in mind, however, that all proteins isolated from biological material are populations of molecules of a different age. Thus, in a preparation of crystalline serum albumin, some of the molecules of the crystal may be 1 or 2 hours old, whereas others may be molecules formed many weeks previously. It is quite logical, therefore, to expect a distribution function of protein molecules of different age. This does not necessarily prove that old molecules are different from young molecules; however, it is known that proteins, if stored in the laboratory in the cold or at body temperature, undergo slow inactivation, i.e., changes in their biological properties. Obviously, these are paralleled by changes in the conformation of the genuine molecules. It would not be surprising, therefore, if old molecules differed from younger molecules of the same "protein species" by slight changes in their conformation.

Other changes may be caused by differences in the site of formation; thus hemoglobin formed in the bone marrow is different from hemoglobin formed in the liver of the human fetus. In some instances, there may be changes in the conformation and also slight changes in the primary structure, such as replacement of an amino acid or formation of dithio bonds from sulfhydryl groups. In addition to these differences brought about by intrinsic factors (age, site of formation), there may be differences due to the interference of external factors (abnormal temperature, poisons, antigens, deficiency of certain nutrients). Antibody formation according to this view, is not an abnormal phenomenon, but a process well within the normal variability of the blood serum γ-globulins.

All the proteins mentioned in the preceding paragraph are soluble substances, some of them obtainable in the crystalline state. Although some of them may act as enzymes, others as antibodies or hormones, they are not animated. They have no metabolism, they do not grow, nor do they reproduce themselves. They are probably nothing but products or fragments of larger proteins that form the true structural substrate of living matter. Life, and its principal phenomena, growth and reproduction, are unimaginable without a structural substrate. This substrate is, most probably, formed by long, threadlike molecules of proteins. Similar molecules are present in keratin or collagen fibers. Their molecular weight seems to be much higher than that of the soluble, crystallizable proteins. Threads of such "superproteins" connect, probably, adjacent structural elements within the cell or lead from cell to cell. They may mediate phenomena such as the pairing of chromosomes or the conduction of stimuli from their origin to adjacent cells. Breakdown of these long chains of superproteins may then give rise to fragments of slightly different types, causing heterogeneity thereby.

Whatever the reason for this heterogeneity, the purely chemical concept of unalterable uniform protein molecules must be abandoned and the biological or biochemical concept of variable protein species, similar to animal or plant species, must be accepted. Each protein species may consist of a family of closely related individual molecules differing from one another by properties that depend on the history of the molecule, its age, its site of formation, and on its exposure to various extrinsic factors.

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THE HETEROGENEITY OF SERUM GLOBULINS

(INVITED DISCUSSION)

FRANK W. PUTNAM
Department of Biochemistry, and Argonne Cancer Research Hospital,
University of Chicago [1,2]

In his conclusion, Dr. Haurowitz brought out an important concept that merits further consideration, namely, the microheterogeneity of proteins. Ten years ago, protein chemists were convinced that many proteins had been prepared in a quite homogeneous form as judged by physicochemical criteria. However, Dr. Alberty and others have now shown (by such methods as boundary spreading measurements) that no protein as yet can be considered truly homogeneous. In regard to chemical structure, Sanger's brilliant elucidation of the complete structure of insulin provides the firmest support yet for the concept that proteins are chemical entities with explicity defined sequence and structure. Yet there is new evidence that biological activity need not be associated with an invariant structure, i.e., the finding of eight corticotropins and of two vasopressins, as well as the species differences in insulin. All these results suggest the need for further study of chemical microheterogeneity.

One approach to the problem of chemical homogeneity lies in N-terminal amino acid analysis, for the number of end groups per mole of proteins should be integral, nonstoichiometric amounts suggesting impurities. This will be a discussion of the application of this method to a study of the heterogeneity of serum globulins, for, as Dr. Haurowitz emphasized, both physicochemical and immunological findings

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[1] Present address. Department of Biochemistry, College of Medicine, University of Florida, Gainesville.

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have indicated that there is a spectrum of normal globulins and of antibodies.

In work begun at Cambridge (Putnam, '53), and independently reported by McFadden and Smith ('53), it was found that there are approximately 1 mole of N-terminal aspartic acid and 1–2 moles of N-terminal glutamic acid per mole of normal, pooled human γ-globulin. The first samples investigated proved to be heterogeneous both electrophoretically and by the end-group method, i.e., in the latter analysis, several additional residues were found in fractional quantities.

TABLE 1

N-Terminal groups of normal human gamma-globulin and of myeloma globulins

PROTEIN	pI	MOBILITY (pH 8.6)	S_{20} S	Asp	Glu	Leu	Ser	Ala
					moles/160,000 *g*			
Normal γ_2	7.2	—1.3	6.6	1.12	1.75		0.11	
Normal II-3	6.85			0.74	0.72		0.11	
Normal II-1,2	7.3			0.78	1.37		0.10	
Myeloma Type I	6.7	—0.76	6.6	0.04	0.08	2.02	0.15	
Myeloma Type II	> 6.4	—1.1	6.6	2.04				
Myeloma Type III	< 6.7	—2.4	6.1,[b] 9	2.05	2.65			
Myeloma Type IV		—0.8	6.4		2.65			
Myeloma Type V	6.7	1.18[a]	6.2	0.49	0.55			0.66

[a] Heterogeneous.

[b] Major component.

Even highly purified subfractions of γ-globulin contained about 0.1 mole of serine, and the ratio of N-terminal glutamic acid to aspartic acid varied from 1.0 to 1.7 in different subfractions. These results are illustrated in table 1, which contains data representative of analyses of many protein preparations. For example, in normal fractions II-3, and II-1,2 (independently analyzed by Smith and McFadden, '53) the molar ratio of end groups rises from 1 to almost 2. This suggests that there are at least two structurally different types of γ-globulin that can be partially separated by the fractionation process. It is interesting to observe that Porter ('50) has demonstrated that both the normal γ-globulin and antiovalbumin of the rabbit contain a single N-terminal residue (alanine), whereas, McFadden and Smith ('53) report that beef γ-globulin and horse γ-globulin have some 5–6 N-terminal residues, none present in stoichiometric proportions.

Entirely different results are obtained when N-terminal amino acid analysis is applied to the pathological globulins that can be isolated in highly homogeneous form from the individual sera of patients with multiple myeloma. These proteins can be grouped into various types according to isoelectric point, ultracentrifugal pattern, and antigenic determinants. Many of the proteins have electrophoretic properties falling within the wide range recorded for γ-globulins, but the myeloma globulins differ from the normal protein in their greater electrophoretic and ultracentrifugal homogeneity, as well as in certain antigenic features (Putnam, '55). End-group analysis similarly demonstrates that these proteins are more homogeneous chemically than normal γ-globulin and that they differ structurally from the normal protein.

Data obtained from the analysis of twenty myeloma globulins are summarized in table 1. On the basis of amino end-group analysis, five types were found — only one type occurred in the serum of each patient. Type I (of which there are two examples) are N-leucyl globulins, for these proteins contain two residues of N-terminal leucine and only minor amounts of N-terminal dicarboxylic acids that are attributable to the presence of normal γ-globulin. Since leucine is not detectable as an amino end group in normal γ-globulin, the N-leucyl globulins are structurally abnormal human globulins. The second type of myeloma globulins may be called N-aspartyl globulins (five specimens), for these proteins have two residues of N-terminal aspartic acid and are either devoid of or contain only minor amounts of N-terminal glutamic acid. A third group of myeloma globulins is comprised of three specimens containing both dicarboxylic acids in the N-terminal position, but in twice the normal amount. The fourth type — N-glutamyl globulins — has essentially only glutamic acid in the N-terminal locus, but the number of amino end groups varied from three to five in the five specimens analyzed. Lastly, there is a group of proteins heterogeneous both by physicochemical criteria and in N-terminal amino acid analysis. No strict correlation exists among the classifications made by N-terminal amino acid content, physicochemical properties, or antigenic type.

From Dr. Haurowitz's point of view, it is possible that in certain circumstances individuals may synthesize new or different proteins. This does seem to be the case in this disease. In eight patients studied who produced a serum cryoglobulin (protein that gels on cooling), each cryoglobulin proved different from normal serum globulins in two or more properties. Moreover, only two of the cryoglobulins were similar in all properties examined (Putnam, '55).

Dr. Haurowitz has presented a very challenging concept of microheterogeneity of proteins. Although I do not subscribe to it fully, I am pleased to have had the opportunity to present the evidence for the heterogeneity of human serum globulins and for the synthesis of new types in disease.

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MICROHETEROGENEITY OF PROTEIN MOLECULES

(INVITED DISCUSSION)

SOL SPIEGELMAN
University of Illinois, Urbana

This idea of heterogeneity is a very exciting one, particularly to those of us who are concerned with the question of how enzyme proteins are put together. It is directly relevant to the question of the relation between the genetic structure of a cell and the kinds of enzyme proteins that can be made.

As I understand Dr. Haurowitz, he has proposed at least three specific mechanisms that can provide a basis for finding heterogeneity in a sample of protein: age (i.e., both old and young proteins may be present in the sample), place of origin (e.g., some are made in the liver, others in bone marrow), and preparative artifacts.

I should like to add one that is implicit in the listed categories and is to us the most interesting; namely, heterogeneity in formation. That is, can the same site produce a variety of distinguishable protein molecules?

It is clearly difficult to obtain any definitive evidence on heterogeneity with metazoons, in which the first two sources of heterogeneity mentioned are difficult to eliminate. Log-phase microbial populations would seem to be a more suitable experimental material since questions of differences in age and places of origin would be minimized. Of course the problem of preparative artifacts is not obviated. Some years ago the question was raised in our laboratory as to whether the nature of the inducing agent modifies in any way the specificity and property of the enzyme being synthesized. The question is relevant since one and the same enzyme activity can be induced by a variety of agents. In the instance to be used as an example, an α-glucosidase can be induced in yeast by means of such compounds as maltose, α-methylglucoside, and turanose. The first results obtained were most exciting since, on isolation, the enzyme induced by maltose possessed a different ratio of maltose to α-methylglucoside hydrolizing ability when compared to the protein that was isolated when α-methylglucoside was employed as the inducer. Since the induction procedure does not change the genetic structure of the cells, this was apparently an instance of modification of enzyme specificity against a constant genetic background.

There was, however, one possibility that we laughingly entertained; namely, that the cell might produce an enzyme different from the usual maltose, and which would split the synthetic analog of maltose, α-methylglucoside, faster than the substrate. If this were the case, the differences in activity ratios could be simply a reflection, not of the production of variants of one species of protein molecules, but of mixtures of two species, each made by a different site. This possibility turned out to be correct. It proved easily possible to separate and demonstrate the existence of a new protein virtually completely specific for the synthetic α-methylglucoside and possessing very little ability to split maltose (Spiegelman *et al.,* '50). In addition, it was possible to exhibit the existence of a specific genetic component necessary for the synthesis of this enzyme (Hestrin and Lindegren, '51). Once this gene is removed by genetic crossing, a cell can be obtained that, when exposed to maltose, α-methylglucoside, or turanose, makes only one enzyme molecule type, as determined by specificity ratios and other devices.

Thus, even with a uniform population of cells in which the question of age and place heterogeneity might not seem relevant, it remains true that any heterogeneity found need not be attributed to errors at the enzyme-forming site. There may exist within this uniform population of cells different enzyme-forming sites, one of which is capable of synthesizing one enzyme molecule and another a related but distinguishable one.

The evidence for heterogeneity presented by both Dr. Putnam and Dr. Haurowitz is not sufficiently conclusive to explain heterogeneity of the activity of one enzyme or protein-forming site in making a molecular species. As Dr. Putnam pointed out, Sanger's work is probably the most convincing evidence that (1) a collection of protein molecules is uniform, and (2) his data are not the result of a statistical norm. But again, the evidence is not completely compelling. The number of peptides required to define with certainty the amino acid array is really not sufficient to eliminate the possibility of a fair degree of statistical variation. It would thus appear that this question of microheterogeneity at the level of a protein-forming site is still an open one.

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HAUROWITZ: Gamma-globulins do not crystallize. This indicates that they are heterogeneous, as pointed out by Dr. Putnam.

I agree very much with what Dr. Spiegelman said. When I spoke of heterogeneity by site of formation, I did not want to limit that to different organs. Different subcellular sites may just as well be responsible for the formation of heterogenous proteins.

HARKER[3]: I want to bring up another point very early in the meeting — one related to Dr. Haurowitz' presentation — and this is the Pauling-Corey helix. Unquestionably, the unstretched and stretched forms of keratin have, respectively, the α-helix and pleated sheet structures of Pauling and Corey. Collagen, on the other hand, has a structure that has not yet been predicted, but that is probably quite simple. No doubt it will be necessary to abandon the idea of the structural equivalence of the amino acid residues; there is no compulsion on nature to construct proteins only according to this principle.

It is my belief that the structures of globular proteins are not necessarily constructed of rod-like entities, such as the Pauling-Corey helices, although this is often said by crystallographers of the Cambridge school. The evidence for such structures is far from convincing, being based on so-called Patterson maps derived from the intensities of X rays diffracted from crystals of these proteins. It is the nature of such maps to show rows of high-density regions that can be interpreted as the vector images of rods, whether or not rods actually exist in the structure of the crystal. The projected electron densities of two protein crystals — hemoglobin and ribonuclease — have been computed. In neither of these is there any evidence of rod-like structure.

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[3] David Harker, Polytechnic Institute of Brooklyn.

HAUROWITZ: The assumption of regular helical structures in proteins is of fundamental importance. Its fascinating power is reminiscent of that of Svedberg's units of molecular weight 17,000, and Bergmann's periodicity hypothesis, although the helices may be based on better experimental evidence. Does the 1.5 A reflection not prove the helical structure in globular proteins?

HARKER: The 1.5 A reflections are almost, if not quite, impossible to detect on the diffraction patterns of crystalline hemoglobin. I believe that they will be found of no significance when the structure of hemoglobin is finally worked out.

THORBECKE[4]: Is there any normal γ-globulin? Are not all γ-globulins antibodies?

HAUROWITZ: You may be right, since newborn animals have no γ-globulins. They all may be formed in response to stimuli.

SCHWERT[5]: I should like to take the skeptic's side in arguing microheterogeneity. If we accept the view that chymotrypsinogen is a highly homogeneous protein, as evidenced by reversible boundary spreading measurements of Alberty and by column chromatography, it appears that activation of chymotrypsinogen by trypsin, a hydrolytic process, yields α-chymotrypsin, a protein that is quite heterogeneous according to end-group determinations. However, as Jansen and Desnuelle and their coworkers have shown, treatment of α-chymotrypsin with DFP yields a product that is much more homogeneous. Since traces of firmly bound peptides appear to be released when α-chymotrypsin is converted to DP-α-chymotrypsin, might not microheterogeneity arise from protein small peptide interactions rather than from primary differences in covalent structure?

HAUROWITZ: This may be true for chymotrypsin, but certainly not for the heterogeneity of antibodies differing from one another by higher or lower affinity to the determinant group of an antigen.

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[4] Geertruida J. Thorbecke, Notre Dame University.

[5] George W. Schwert, Duke University School of Medicine.

Borsook[6]: One of my colleagues, Dr. Sweet, found that lysine was incorporated by the ε-amino group, some of it also by the carboxyl group.

Haurowitz: Linkage of lysine by the ε-group has also been observed in bacitracin. Upon reinvestigation of the old problem of plastein formation, we found that it consisted essentially of transpeptidation. Thus a peptic digest of ovalbumin, after treatment with chymotrypsin, will incorporate the phenylalanyl residue of added radioactive phenylalanine ethyl ester, but will not incorporate free phenylalanine. The total number of free amino or carboxyl groups does not change in this reaction.

Borsook: I think it was Virtanen and his group who showed that only peptides above a certain size can go to form plasteins. Every worker on this subject, with one exception, has found that plasteins cannot be made from free amino acids. The incorporation of esters of phenylalanine is essentially Brenner's experiment. He started with a single amino acid ester and with chymotrypsin and got very large molecules. This is a transpeptidation.

On the other hand, I do not think plastein formation in the main is a transpeptidation because there is a reduction in the number of free amino and free carboxyl groups when plasteins are formed. This has been debated but a number of people agree on it, including Virtanen, whose measurements are the latest. With simple transpeptidation, there would be no change in the number of free amino and free carboxyl groups.

The data indicate that, with large enough peptides, the free energy formation of the bond will be so small that some peptide linkage between them can occur by mass action, especially if the resulting product precipitates out.

Halvorson[7]: In recent studies on α-glucosidases similar to those discussed by Dr. Spiegelman, we observed at least two separate enzymes inducible by maltose. These enzymes, which can be genetically separated in different strains, differ not only in their substrate specificity toward glucosides, but also in their electrophoretic mobilities. Both enzymes can be induced by a variety of α-glucosides.

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[6] Henry Borsook, California Institute of Technology.

[7] H. O. Halvorson, University of Michigan.

FRAENKEL-CONRAT[8]: I wish to remind Dr. Haurowitz that the high binding affinity of serum albumin cannot be an ionic phenomenon only, since many nonpolar compounds are also firmly bound. I am sure he agrees with me that more complex interactions are involved.

I should like to express the opinion that microheterogeneity is not a very useful concept. For chemists, it seems to be a more satisfying working hypothesis to assume that proteins can be homogeneous. Although a certain range of individual molecules may occur in a given sample of such a complicated hydrogen-bonded polymer, these probably differ by only slight degrees of denaturation, aggregation, and binding of ions. We are all aware that there are families of closely related but chemically different proteins, such as the hemoglobin and antibodies. And many proteins believed to be homogeneous have proved to contain two or more molecular species when better methods became available. In principle, however, the concept is still to be favored that most proteins can be purified to consist largely of molecules of one species.

HAUROWITZ: I think that the closer the approach to the ''living'' state of a protein the less crystallinity and the more heterogeneity will be found.

ROEDER[9]: When age is regarded as a factor in producing microheterogeneity, it must be taken into consideration that not all proteins are affected by aging procedures. Nucleoproteins, in particular, and intracellular proteins subject to continuous repair, in general, may differ greatly from serum proteins.

In metazoans, it is possible to demonstrate that, in a population of aging cells, a change in environment will call forth a duplicate of an earlier type of protein. Arginine added to the chick embryos induces the formation of arginase, even in the period when the embryo is uricotelic, and the presence of the enzyme is disadvantageous. This production indicates that the production of cellular protein is not affected to the same extent as in serum globulin. The cell, as a continuous repairing unit, may force stability on proteins; this stability could easily be lacking in circulating extracellular species.

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[8] H. Fraenkel-Conrat, University of California.

[9] Martin Roeder, Woman's College of the University of North Carolina.

SPIEGELMAN: This cannot be accepted as conclusive evidence against age playing a role. For example, selection of a DNA nucleoprotein as a sample protein presents a rather formidable difficulty, since DNA is a self-duplicating unit that has, at least potentially, the possibility of self-repair. Aging phenomena might well disappear with units of that kind.

KAUZMANN[10]: In regard to Dr. Fraenkel-Conrat's remarks about purity, it seems to me that it would be wise to anticipate a certain amount of inherent variability in composition from one molecule to another in even the "purest" protein preparations. A trypsin molecule should not change its activity very much if, say, leucine were to replace isoleucine at some point along the polypeptide chain, especially if the site of replacement is not close to the active region of the molecule. Similarly, aspartic acid might replace glutamic acid and tyrosine might replace phenylalanine, and so on. How can we be sure, therefore, that in the synthesis of proteins, a certain amount of such substitution does not actually occur? It it does occur, have we any right to say that each "pure" protein molecule must contain the same whole numbers of amino acids, all located at definite points along the chain? I can see no *a priori* necessity for believing that, in a molecule like trypsin, which contains something like 400 amino acid residues, every one of the 400-odd sites in the chain must be occupied in exactly the same way in every enzymically active molecule. Until there is definite evidence that this kind of reproducibility actually occurs, it is more reasonable to expect that it does not exist.

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[10] Walter Kauzmann, Princeton University.

In many minerals (e.g., the feldspars), it is possible to replace one ion by another over a wide range of compositions, resulting in a family of minerals having closely similar properties. In such cases, the concept of "purity" with respect to the substituting ions proves to be of little use. It is quite possible that the similar possibility of the limited replacement of some amino acid residues by others will impose practical limitations on present concepts of the purity of proteins.

HAUROWITZ: It may well be, as Dr. Kauzmann said, that heterogeneity is brought about by enzymes. Indeed, enzymes are much less specific than we thought, as shown by the example of chymotrypsin, which splits ester bonds just as well as amide bonds.

HARKER: The geometric structures of molecules are not always changed by changing the chemical nature of some of their atoms; for instance, ferric ion can be replaced by aluminum ion, or four aluminum ions by three silicon ions, without affecting the structure of a mineral. These atoms occupy the same positions as those they replace and have the same structural significance.

The same kind of thing can happen in the molecules of globular proteins. Thus an amide group can replace a carboxyl group without changing atomic positions significantly. I believe this kind of difference accounts for the two varieties of ribonuclease that can be separated chromatographically from a specimen giving perfect crystals.

The X-ray diffraction patterns of these crystals are magnificent, and indicate an essentially perfect repetition of atomic positions from one molecule to the next. This repetition does not rule out the substitution of an occasional atom by one of another kind, or by a vacancy.

BECKER[11]: Dr. Feinberg and I, in collaboration with Drs. Peterson and Sober, have fractionated human serum albumin by means of the cellulose ion-exchange columns developed by Drs. Peterson and Sober and have been able to show that albumin fractions that are homogeneous in the ultracentrifuge and electrophoretically *p*H 8.6, differ in their tyrosine and tryptophan content (the only amino acids examined) and quantitatively in their behavior in the precipitin reaction. Other fractions have been obtained which differ in their carbohydrate content from the starting material and also in the amount of aggregated material they contain and, even more strikingly than the previously mentioned fractions, in their immunochemical behavior.

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[11] Elmer Becker, Walter Reed Army Medical Center.

HAUROWITZ: Cannot such exchange columns cause denaturation?

BECKER: Two of our fractions show no trace of aggregation in the ultracentrifuge; there are definite differences between them in their content of tyrosine and tryptophan and in their immunochemical behavior. It is difficult to believe that these chemical differences could be caused by denaturation; there is no evidence of denaturation. Yet the point raised by Professor Haurowitz is a real one, which will require further work for definite settlement.

EDELHOCH[12]: I might mention the beautiful work of Dr. Hughes, who showed that there are two fractions of normal crystalline serum albumin, of which about 70% is reactive toward mercury and 30% is inactive. Presumably, the difference between the two species resides in the availability of a sulfhydryl group.

HERRIOTT[13]: A restatement of the questions or proposition under consideration would be helpful at this point. If I understand the problem to be "How to purify any protein so that it is homogeneous by all tests," then the answer is almost certainly that no one procedure will accomplish this.

HAUROWITZ: We have to differentiate between two things. One is the different shape of molecules like ethane or rubber, which, owing to thermic motion, will exert rotation around the long axis of their carbon chains and assume different shapes at different times. The other is the different shape of globular protein molecules, which, owing to their three-dimensional structure, cannot undergo periodical changes in their structure but are more or less rigid. Only this type of heterogeneity is of biological importance.

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[12] H. Edelhoch, University of Kansas.

[13] R. M. Herriott, The Johns Hopkins University.

SPIEGELMAN: What we want to know, regardless of these other characteristics such as pH, mobility, or solubility, is the range of permissible error when one unit of protein-forming mechanism (whatever it is called) is putting the molecule together.

As a result of the calculations by Gamow and others, and if the Watson-Crick model is accepted as a suitable code, twenty or more amino acids can be specified without any difficulty. The DNA molecule certainly contains that much information. But, in a particular array of purines and pyrimidines, say a set of four, so joined as to specify, e.g., leucine, how frequently if ever is it replaced by another amino acid?

I must confess that I cannot accept thermodynamic calculations on this point because too little is known about the actual concentrations at the site of synthesis to make even remotely reliable estimations. They could err by orders of magnitude. It is now known that, as far as informational content is concerned, whether it is transferred to RNA or acts directly, the composition of DNA is adequate to make the kind of distinctions to obtain complete precision. The kind of information wanted is whether it really *is* complete.

DOUNCE[14]: Information in three categories is probably needed: (1) the amino acid composition, (2) the sequences of amino acids in the peptide chains, and (3) coiling. Of course, other things also need to be known, such as how many peptide chains there are, but these are secondary. It seems to me that breaking the problem down into these three categories immediately reveals what must be known for interpretation of heterogeneity, which is discovered by physicochemical methods.

The insulin molecule might be mentioned in this connection. I wonder how much microheterogeneity, for instance, of any type there can be in the insulin molecule if we accept the structure. I do not see how there can be any, but this is only one kind of molecule. It is possible, of course, that there are some errors in the work and the structure really is not quite so complete as we think.

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[14] A. L. Dounce, University of Rochester.

SPIEGELMAN: As far as insulin is concerned, it can be said, for example, that in hog and beef, there is replacement of one amino acid by another — valine by something else.

DOUNCE: Yes, in different species.

SPIEGELMAN: That is right. There is no information as to whether in the same species there is possibility of replacement in the insulin.

DOUNCE: I meant my remarks to refer only to proteins within a given species.

HAUROWITZ: As far as I know, Craig found heterogeneity in insulin of a single animal species.

THE BIOSYNTHESIS OF PEPTIDES AND PROTEINS[1]

HENRY BORSOOK
Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena

ONE FIGURE

INTRODUCTION

In the present state of the problem of the mechanism of peptide and protein synthesis, a hypothesis is needed at the level of reaction mechanism in terms of chemical formulas. Very probably the clearer and the more detailed picture attempted now the more clearly and the more definitely will it be demonstrated to have been wrong.

This review is such an attempt. The hypothesis is as follows. There are, viewed in the large, three steps in the synthesis of protein: first, activation of the carboxyl group of free amino acids; second, transport of the amino acids so activated to a nucleic acid template where they are arranged in a specific sequence. On this template the amino acids are linked by peptide bonds and then the protein molecule is peeled off the template; this is the third step. Either in the course of its peeling off the template or shortly afterward, the specific coiling of the protein occurs spontaneously, governed by its amino acid pattern. Or it is modified to another stable pattern by, for example, an agent such as an inducer in the formation of an adaptive enzyme, or an antigen in the formation of an antibody. Since small peptides and quasipeptides have, as it were, only a rudimentary amino acid pattern, certain features of protein synthesis are telescoped or missing in their synthesis.

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Carboxyl groups are activated in a number of different types of enzymic reactions. In the hypothesis to be presented here, only the ATP-dependent [2] carboxyl group activation is envisaged. Other types of carboxyl group activation are mediated by proteases, peptides, and transferases and have been called replacement or transfer reactions. Their essential feature is conservation of some of the energy of the bond that is cleaved by activation of the carboxyl group of the cleavage product, presumably, by combination with the enzyme; the enzyme may be displaced by water, ammonia, hydroxylamine, hydrazine, or the amino group of another amino acid or peptide. Nothing is known regarding the role of such transfer reactions in the biosynthesis of proteins and peptides; it seems likely that they do occur *in vivo*. It is certain they are not involved in the first step by which free amino acids are built into peptides and protein, where there is a net gain of peptide bonds (Borsook, '53). The picture to be presented of the mechanism of protein synthesis is an extension and elaboration of the mechanisms by which quasipeptides, amides, and small peptides are synthesized. Since the main features of these mechanisms are now clear, they will be considered first.

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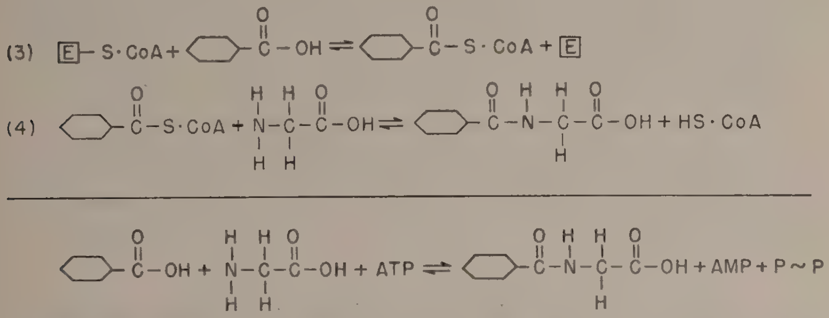
[2] Abbreviations in this article: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A; AMP, adenosine monophosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

SYNTHESIS OF HIPPURIC ACID

The steps in the synthesis of hippuric acid are as follows:

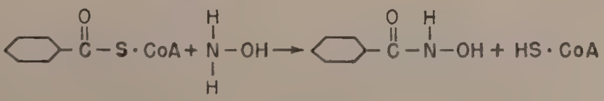
(1) $\boxed{E} + \mathrm{ATP} \rightleftharpoons \boxed{E}-\mathrm{AMP} + \mathrm{P}\sim\mathrm{P}$

(2) $\boxed{E}-\mathrm{AMP} + \mathrm{HS\cdot CoA} \rightleftharpoons \boxed{E}-\mathrm{S\cdot CoA} + \mathrm{AMP}$



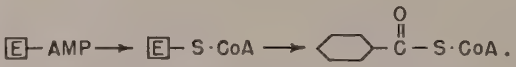
The synthesis of hippuric acid from benzoic acid and glycine represents a gain in free energy of the same order of magnitude as that of a dipeptide bond, 2500–4000 calories (Borsook and Dubnoff, '40). Accordingly, the enzymic synthesis of hippuric acid must be coupled with a free energy donor; Cohen and McGilvery ('46, '47a, b) and Borsook and Dubnoff ('47) found that the reaction was dependent on ATP. Chantrenne ('51) demonstrated that CoA is required, and then Schachter and Taggart ('53, '54a, b) proved that it is benzoyl-CoA that reacts with glycine to form hippuric acid. The steps by which benzoyl-CoA is formed are taken directly from the mechanism given by Jones *et al.* ('53) for the formation of acyl-CoA. Schachter and Taggart ('54a) showed that the benzoyl radical may be replaced, in conjugation with CoA, by a variety of aliphatic and aromatic acyl groups, but the enzyme is specific for glycine, and named it, therefore, glycine *N*-acylase. The enzyme for the synthesis of the acyl-CoA is presumed to be identical with the fatty acid-activating enzyme.

Benzoyl-CoA reacts nonenzymically with hydroxylamine to form benzoyl hydroxamate.



Hydroxamate formation is a mode of trapping activated carboxyl groups.

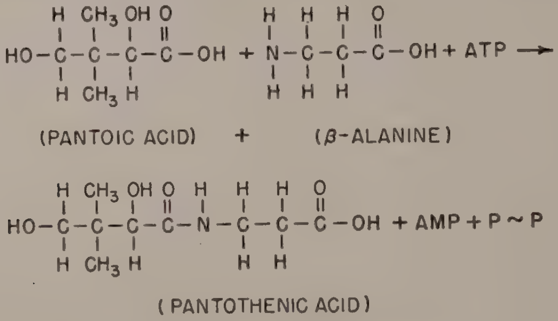
It will be noted that the carboxyl, not the amino, group entered into the quasipeptide bond that was activated. The energy for its activation came from one of the pyrophosphate bonds of ATP conserved, in part, in transmission through the transfers,



It is noted also that the ATP is cleaved to AMP and inorganic pyrophosphate.

SYNTHESIS OF PANTOTHENIC ACID

Maas and Novelli ('53) showed that a similar cleavage of ATP promotes the synthesis of pantothenic acid, although CoA does not appear to be involved; the enzyme was obtained from *Escherichia coli*. The over-all reaction is as follows:



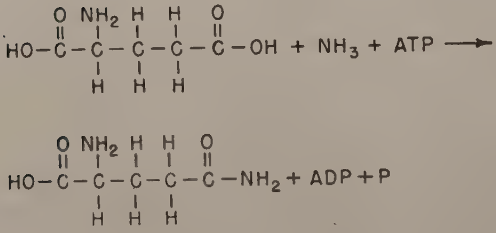
$$HO-\underset{H}{\overset{H}{C}}-\underset{CH_3}{\overset{CH_3}{C}}-\underset{H}{\overset{OH}{C}}-\overset{O}{\overset{\|}{C}}-OH + \underset{H}{\overset{H}{N}}-\underset{H}{\overset{H}{C}}-\underset{H}{\overset{H}{C}}-\overset{O}{\overset{\|}{C}}-OH + ATP \longrightarrow$$

(PANTOIC ACID) + (β-ALANINE)

$$HO-\underset{H}{\overset{H}{C}}-\underset{CH_3}{\overset{CH_3}{C}}-\underset{H}{\overset{OH}{C}}-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-\underset{H}{\overset{H}{C}}-\underset{H}{\overset{H}{C}}-\overset{O}{\overset{\|}{C}}-OH + AMP + P\sim P$$

(PANTOTHENIC ACID)

SYNTHESIS OF GLUTAMINE



$$HO-\overset{O}{\overset{\|}{C}}-\underset{H}{\overset{NH_2}{C}}-\underset{H}{\overset{H}{C}}-\underset{H}{\overset{H}{C}}-\overset{O}{\overset{\|}{C}}-OH + NH_3 + ATP \longrightarrow$$

$$HO-\overset{O}{\overset{\|}{C}}-\underset{H}{\overset{NH_2}{C}}-\underset{H}{\overset{H}{C}}-\underset{H}{\overset{H}{C}}-\overset{O}{\overset{\|}{C}}-NH_2 + ADP + P$$

Speck ('47, '49a, b) and Elliott ('48a, b) discovered enzyme systems that can synthesize glutamine from glutamic acid and ammonia according to the preceding reaction. The γ-carboxyl of glutamic acid is the only acceptor of the free base, which may be ammonia, hydroxylamine, hydrazine, or methylamine. Elliott ('53) and later Varner and Webster ('55) purified the enzyme greatly. The synthesizing activity throughout the purification paralleled the transferase activity, which may be represented as

$$\gamma\text{-glutamo}\cdot NH_2 + \overset{*}{N}H_3 \rightleftharpoons \gamma\text{-glutamo}\cdot \overset{*}{N}H_2 + NH_3\ .$$

In spite of this parallel, it is uncertain whether or not the enzyme locus is the same for the synthetase and transferase activities. The synthetase optimum *p*H is 7.5; it is activated more by Mg^{++} than by Mn^{++}, and is activated by cysteine. The transferase optimum *p*H is 6.75; it is activated more by Mn^{++} than by Mg^{++}, is not activated by cysteine, and requires ADP or ATP and phosphate or arsenate. ADP is a better cofactor for the transfer reaction than ATP. AMP is ineffective in both reactions. The same enzyme also catalyzes an arsenate-activated hydrolysis of glutamine, for which Mg^{++} or Mn^{++} and ADP are required; ATP is inhibitory.

Phosphorylated intermediates of glutamic acid have been sought, but no trace of any has been found. Varner and Webster ('55) tested the following possibilities as first steps in the reaction.

$$(1)\qquad \boxed{E} + \text{ATP} \rightleftharpoons \boxed{E}\text{–P} + \text{ADP}$$

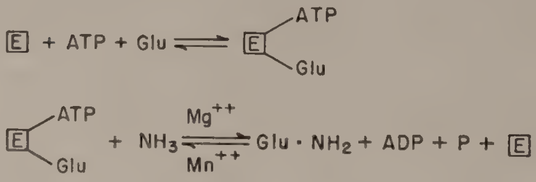
$$\text{or}$$

$$\rightleftharpoons \boxed{E}\text{–ADP} + \text{P}$$

$$(2)\qquad \left.\begin{array}{l}\boxed{E}\text{–P}\\ \quad\text{or}\\ \boxed{E}\text{–ADP}\end{array}\right\} + \text{Glu} \rightleftharpoons \boxed{E}\text{–Glu} + \left\{\begin{array}{l}\text{P}\\ \text{or}\\ \text{ADP}\end{array}\right.$$

If any of these alternatives were correct, in the presence of glutamate and ATP either ADP* or P* should exchange its P* with that of ATP. This was not the case. Such exchange did occur only when and if at least a catalytic amount of NH_3

was added. The reaction mechanism appears to be somewhat as follows:

(1)
(2)


$$\boxed{E} + \text{ATP} + \text{Glu} \rightleftharpoons \boxed{E}\genfrac{}{}{0pt}{}{\text{ATP}}{\text{Glu}} \tag{1}$$

$$\boxed{E}\genfrac{}{}{0pt}{}{\text{ATP}}{\text{Glu}} + \text{NH}_3 \xrightleftharpoons[\text{Mn}^{++}]{\text{Mg}^{++}} \text{Glu}\cdot\text{NH}_2 + \text{ADP} + \text{P} + \boxed{E} \tag{2}$$

When this reaction was carried out in O^{18}-labeled water, no O^{18} was found in any of the participants in the reaction. This finding attests to the correctness of the mechanism as far as it is postulated.

Step (2) alone is the transfer reaction. In it, arsenate can replace P. The over-all reaction is far to the right in favor of glutamine; as a result, only a slight exchange of P* can be demonstrated.

With hydroxylamine, the over-all reaction is even farther to the right than with ammonia. Accordingly, hydroxylamine could replace ammonia in glutamine (the transfer reaction), and no transfer of P* or ADP* into ATP could be demonstrated.

Here again, it is certainly the γ-carboxyl of glutamate that is activated. No CoA is involved. Unlike the syntheses of hippuric and pantothenic acids, the cleavage products of ATP are not AMP and P ∼ P but ADP and P.

Webster and Varner ('55a, b) have found a different but in all respects analogous enzyme for the synthesis of asparagine and asparaginylhydroxamate from aspartic acid and ammonia or hydroxylamine.

SYNTHESIS OF GLUTATHIONE

The enzymic synthesis of glutathione from its constituent amino acids was achieved by Bloch and coworkers and the over-all reactions were demonstrated to be as follows (Bloch, '49; Bloch and Anker, '47; Johnston and Block, '49, '51;

Block *et al.*, '52; Snoke and Bloch, '52; Snoke and Rothman, '51; Yanari *et al.*, '52).

$$(1) \quad \text{Glu} + \text{Cys} + \text{ATP} \xrightarrow{\substack{K^{+} \\ Mg^{++}}} \gamma\text{-Glu}\cdot\text{Cys} + \text{ADP} + \text{P}$$

$$(2) \quad \gamma\text{-Glu}\cdot\text{Cys} + \text{Gly} + \text{ATP} \xrightarrow{\substack{K^{+} \\ Mg^{++}}} \gamma\text{-Glu}\cdot\text{Cys}\cdot\text{Gly} + \text{ADP} + \text{P}$$

One pyrophosphate linkage of ATP is used in the synthesis of each peptide bond. ADP is inhibitory. Apart from ATP, no other coenzyme appears to be required. No phosphorylated intermediates have been found.

In the synthesis of each peptide bond the first step is

$$\boxed{E} + \text{ATP} \rightleftharpoons \boxed{E}\!-\!\text{P} + \text{ADP}.$$

This was established for the formation of the Glu·Cys bond by Webster and Varner ('54) and for the Cys·Gly bond by Snoke ('53). The evidence in both cases was the same. The enzyme was incubated with ATP and ADP^{32} and the ATP became labeled. The sequence of events was, therefore,

$$\begin{array}{ll} (1) & \boxed{E} + \text{ATP} \rightleftharpoons \boxed{E}\!-\!\text{P} + \text{ADP} \\ (2) & \boxed{E}\!-\!\text{P} + \text{ADP}^{32} \rightleftharpoons \boxed{E} + \text{ATP}^{32} \\ \hline & \text{ATP} + \text{ADP}^{32} \rightleftharpoons \text{ADP} + \text{ATP}^{32} \end{array}$$

With $\boxed{E}\!-\!\text{P}$ established as the first step, Webster and Varner ('54) established the next step to be

$$\boxed{E}\!-\!\text{P} + \text{Glu} \rightleftharpoons \boxed{E}\!-\!\text{Glu} + \text{P}.$$

They incubated P* with the enzyme, glutamate, and ATP and found that the ATP became labeled. The reaction sequence was therefore

$$\boxed{E} + \text{ATP} \rightleftharpoons \boxed{E}\!-\!\text{P} + \text{ADP}$$

$$\boxed{E}\!-\!\text{P} + \text{Glu} \rightleftharpoons \boxed{E}\!-\!\text{Glu} + \text{P}.$$

When cysteine was added, less P* was incorporated into the ATP. The final step, therefore, is

$$\boxed{E}\!-\!\text{Glu} + \text{Cys} \rightleftharpoons \boxed{E} + \gamma\text{-Glu}\cdot\text{Cys}.$$

If the reaction mechanism had been

$$\gamma\text{-Glu}\cdot\text{P} + \text{Cys} \rightleftharpoons \gamma\text{-Glu}\cdot\text{Cys} + \text{P}_i$$

no transfer of P* into ATP* should occur in the absence of cysteine and addition of cysteine should increase the phosphate transfer. The reverse occurred (Snoke and Block, '52).

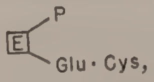
It is interesting that the purified glutamine synthetase enzyme catalyzed the synthesis of γ-glutamylcysteine.

The reaction mechanism appears to be analogous for the synthesis of the Cys·Gly bond of glutathione; but the equilibrium is much farther to the right.

$$\boxed{\text{E}}\text{—P} + \text{Glu}\cdot\text{Cys} \rightleftharpoons \boxed{\text{E}}\text{—Glu}\cdot\text{Cys} + \text{P}$$

$$\boxed{\text{E}}\text{—Glu}\cdot\text{Cys} + \text{Gly} \rightleftharpoons \text{Glu}\cdot\text{Cys}\cdot\text{Gly} + \boxed{\text{E}}\,.$$

From this scheme it would be expected that, when the enzyme is incubated with P*, Glu·Cys, and ATP, the ATP would become labeled. In the experiments of Snoke *et al.* ('53) the result was negative. But Webster and Varner ('55c), using highly labeled P^{32}, did find a low degree of labeling of the ATP when the reaction mixture contained Glu·Cys but not otherwise. Either the equilibrium is very far to the right or the intermediate is



$$\boxed{\text{E}}\begin{matrix}\diagup \text{P} \\ \diagdown \text{Glu}\cdot\text{Cys},\end{matrix}$$

which then reacts with Gly to give Glu·Cys·Gly, $\boxed{\text{E}} + \text{P}$.

The hydroxamate of Glu·Cys can be formed in this reaction, but NH_2OH should be 0.4 *M* to be as effective as 10^{-3} *M* glycine.

No synthesis of glutathione by transfer reactions of the type

$$\gamma\text{-Glu}\cdot\text{Gly} + \text{Cys}\cdot\text{Gly} \rightleftharpoons \gamma\text{-Glu}\cdot\text{Cys}\cdot\text{Gly} + \text{Gly}$$

$$\gamma\text{-Glu}\cdot\text{Cys} + \gamma\text{-Glu}\cdot\text{Gly} \rightleftharpoons \gamma\text{-Glu}\cdot\text{Cys}\cdot\text{Gly} + \text{Glu}$$

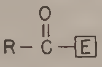
could be demonstrated by Snoke *et al.* ('53) with their purified enzyme. Fodor *et al.* ('53), using a kidney enzyme, did find a synthesis of glutathione to occur by a transfer reaction of the type

$$\text{Cys}\cdot\text{Gly} + \gamma\text{-Glu}\cdot\text{Gly} \rightleftharpoons \gamma\text{-Glu}\cdot\text{Cys}\cdot\text{Gly} + \text{Gly}.$$

There is no utilization of ATP in this mode of glutathione synthesis. There are no data on the biological significance of this reaction.

CARBOXYL GROUP ACTIVATION IN THE SYNTHESIS OF PROTEIN

In the four types of synthesis considered, CoA was involved in only one; in two, ATP was cleaved to AMP and pyrophosphate, and in two others to ADP and orthophosphate. All four had two features in common — it was the carboxyl group that was activated — and this activation was achieved at the expense of one or the other pyrophosphate bonds of ATP. The four syntheses differed in the form of the activated carboxyl group, which was linked to CoA, or directly to the enzyme, or was in an enzyme phosphate complex. In no case was the activated carboxyl group a carboxyl phosphate of the free amino acid.

Also, in all refashioning of new peptide or amide bonds by transfer reactions not involving cleavage of ATP, the evidence is very strong that it is the carboxyl group that is activated by remaining attached to the enzyme. No activation of the amino group is required; it combines with the activated carboxyl group to reconstitute the peptide (or amide) bond simply by displacing the enzyme from the  $R-\overset{O}{\overset{\|}{C}}-\boxed{E}$ linkage.

On the basis of analogy, therefore, the author has suggested that the first step in protein synthesis was activation of the carboxyl groups of free amino acids, utilizing for this purpose one of the pyrophosphate bonds of ATP (Borsook, '54, unpublished [3]). In addition, there was some supporting experimental evidence in the literature. Peterson and Greenberg ('52) had found that ATP accelerated amino acid incorporation into proteins *in vitro* in an enzyme system that was composed of mitrochondria plus a supernatant fraction of rat liver homogenate. Siekevitz ('52) had found evidence of a soluble cofactor produced by mitrochondria, which stimulated amino acid incorporation *in vitro* into rat liver microsomes. He also observed some stimulation by ATP, and was of the opinion that ATP was involved in the formation of the soluble cofactor. Zamecnik and coworkers followed up the observations (Borsook *et al.*, '50a; Hultin, '50) that the microsomes of the liver have the highest rate of amino acid incorporation of any liver fraction and demonstrated that this incorporation requires ATP and a heat-labile, nondialyzable constituent of the supernatant fraction of liver homogenate after it had been centrifuged at 100,000 *g* (Keller, '51; Zamecnik and Keller, '54; Keller *et al.*, '54).

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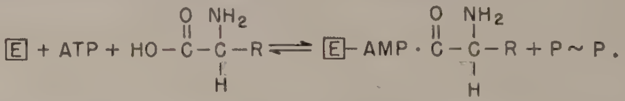
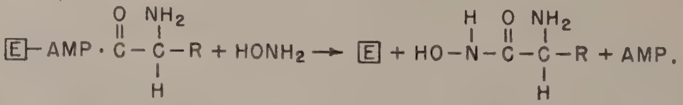
[3] Presented at Biology seminar, Oak Ridge National Laboratory, June 21, 1955.

In Zamecnik's laboratory, Hoagland ('54) has performed experiments that indicate that the heat-labile, nondialyzable fraction of liver supernatant contains an enzyme that, with ATP, activates the carboxyl group of free amino acids. The following reaction mechanism was suggested:



$$\boxed{E} + \mathrm{ATP} + \mathrm{HO}-\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\underset{\underset{\mathrm{H}}{|}}{\overset{\overset{\mathrm{NH_2}}{|}}{\mathrm{C}}}-\mathrm{R} \rightleftharpoons \boxed{E}-\mathrm{AMP}\cdot\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\underset{\underset{\mathrm{H}}{|}}{\overset{\overset{\mathrm{NH_2}}{|}}{\mathrm{C}}}-\mathrm{R} + \mathrm{P}\sim\mathrm{P}.$$

The evidence published so far is the formation of hydroxamate. The mechanism given was



$$\boxed{E}-\mathrm{AMP}\cdot\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\underset{\underset{\mathrm{H}}{|}}{\overset{\overset{\mathrm{NH_2}}{|}}{\mathrm{C}}}-\mathrm{R} + \mathrm{HONH_2} \rightarrow \boxed{E} + \mathrm{HO}-\overset{\overset{\mathrm{H}}{|}}{\mathrm{N}}-\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\underset{\underset{\mathrm{H}}{|}}{\overset{\overset{\mathrm{NH_2}}{|}}{\mathrm{C}}}-\mathrm{R} + \mathrm{AMP}.$$

The main evidence presented in support of the conclusions drawn regarding carboxyl group activation was an increase in the hydroxamate color with iron when amino acids were added to a reaction mixture containing the enzyme, ATP, and other necessary ingredients. It was also stated in the note in which this work appeared that a spot corresponding to leucine hydroxamate was obtained on a filter paper chromatogram. In this laboratory, similar findings were made with the supernatant after centrifugation at 100,000 *g* of lysed rabbit reticulocytes. At present, there are reservations regarding data from these experiments and also Hoagland's. In the colorimetric assay for hydroxamates (Lipmann and

Tuttle, '45), with iron in acid solution (*p*H 0.5–1.5), the color given by amino acid hydroxamate is very much less than that given by, for example, aceto- or succinylhydroxamate. In both these and Hoagland's experiments, the increase in color due to amino acids was very small. In these experiments, the addition of ATP increased the amount of hydroxamate color after 1 hour's incubation, and this color was further increased by the addition of amino acids. An attempt is now being made to obtain more direct evidence by use of labeled amino acids and carrier isolation of the labeled hydroxamate. The tentative findings are positive.

If the interpretation now held very tentatively is confirmed, these findings will constitute direct evidence that amino acid incorporation is based on, and must be preceded by, activation of the carboxyl groups of amino acids. Given activated carboxyl groups of amino acids, the amino groups of other amino acids need no activation for formation of peptide bonds.

Support for the view that the first step in the synthesis of protein is activation of the carboxyl groups of free amino acids by ATP can be found in the many observations that the formation of adaptive enzymes in microorganisms depends on the simultaneous presence of an energy source and free amino acids (Spiegelman and Dunn, '47–'48; Sussman and Spiegelman, '50; Halvorson and Spiegelman, '53; Gale, '51b; Gale and Paine, '51; Gale and Folkes, '53d, e, '54; Jebb *et al.,* '50; Pinsky and Stokes, '52). This energy may be generated either aerobically or anaerobically (Spiegelman and Dunn, '47–'48; Jebb *et al.,* '50; Pinsky and Stokes, '52). Repeatedly, in reports of this work it is emphasized that free amino acids (or a nitrogen source readily convertible to amino acids), an energy source (or active metabolism), and the inducer of adaptation must be present together. Conversely, inhibitors of respiration, and even more significantly, inhibitors of oxidative phosphorylation, thus inhibiting formation of ATP, promptly inhibit formation of adaptive enzymes (Sussman and Spiegelman, '50; Gale and Paine, '51; Jebb *et al.,* '50). This is the situation in microorganisms

(Keeler, '51; Zamecnik and Keller, '54; Keller *et al.,* '54). Gale and Folkes ('54) showed that the same occurs in disrupted cells of *Staphylococcus aureus,* i.e., in intracellular particles of these organisms.

In the earliest studies of protein metabolism in mammals, nearly a half-century ago, it was found that when the amount of protein eaten tended to be short, carbohydrate had a sparing action on the amount of nitrogen lost from the body (Cathcart, '09–'10). Geiger ('48a, '50a) and Munro ('49) showed that, to exert this effect, the carbohydrate must be fed at the same or within a short time of the protein.

TABLE 1

Effect of glucose on protein synthesis in rabbit reticulocytes in vitro

ADDITION TO REACTION MIXTURE	TIME *(hr)*							
	1	2	3	4	1	2	3	4
	Incorporation				Effect of glucose			
	(cts/min)/mg				*(cts/min)/mg*			
Blank	3.32	4.75	6.38	7.43				
+ Glucose	3.12	5.03	6.89	7.72	— 0.20	0.38	0.51	0.29
+ Amino acids	14.59	22.94	25.78	26.01				
+ Glucose + amino acids	15.01	26.15	34.05	36.84	0.42	3.21	8.27	10.83
					Percentage of total incorporation			
					2.7	12.2	24.2	29.3

The interpretation is that during the feeding of carbohydrate there is an increased generation of ATP at the same time that the blood and tissue amino acid concentration is increased. Consequently, the concentration of activated amino acids is increased and this concentration, all other things being equal, governs the rate of protein synthesis. The protein-sparing action of carbohydrate means a faster rate of protein synthesis.

Table 1 shows the stimulating effect of carbohydrate metabolism on protein synthesis *in vitro.* These data were obtained with rabbit reticulocytes. The protein synthesized was mainly (90% or more) hemoglobin. The cells contain some sugar initially; hence, added carbohydrate became effective only after this sugar was used up (after at least 2 hours). Even then the added sugar has no effect unless the conditions are suitable for a very high rate of protein synthesis, i.e., optimal amino acid mixture and an excess of iron. The high rate of protein synthesis accelerates the utilization of the original sugar in the cells and then, the data show, added sugar is necessary to maintain the high rate of protein synthesis.

It seems likely that the phenomenon known as the ''specific dynamic action'' of proteins is based on an increased rate of generation and utilization of ATP evoked by an increase in the concentration of amino acids in the blood and tissues. Shortly after a protein meal is eaten and for a few hours thereafter, there is a considerable increase in metabolism; it may be as large as 25–30%. This increase is three to five times as great as after eating an isocaloric quantity of carbohydrate or fat. The rise and subsequent fall in metabolism follows fairly closely the rise and fall of amino acid concentration in the blood and, presumably, in the tissues (Seth and Luck, '25). There is increased utilization of ATP in the increased activation of carboxyl groups, in increased urea formation and increased protein synthesis; there is also increased oxidative deamination; hence, the specific dynamic action of protein (Borsook, '36).

THE QUESTION OF PEPTIDE INTERMEDIATES

After the amino acids have been activated, the next step is their transport in the activated state to the matrix on which the specific pattern of amino acids is formed, to the template, as it has been called. No energy is gained by making peptides at this stage. An activated carboxyl group on each peptide would be needed for attachment to the template and for the making of the peptide bonds by which the protein is finally put together. There is much more flexibility for the making of many different amino acid patterns in doing so from activated single amino acids than from activated peptides.

If proteins are made directly from amino acids without the intervention of peptides, then, if the amino acids are labeled, the same amino acid should have the same specific activity at all loci in the protein molecule. If there are peptide intermediates, some peptides might be expected to be in the cell before the advent of a labeled amino acid, and these cells would contain the same amino acid unlabeled. In the synthesis of the protein, the peptide would go to form one portion of the molecule; the free labeled amino acid would be used in formation of other portions. The result would be that, until the preexisting peptide was largely used up, there would be unequal labeling of an amino acid at different loci in the protein molecule. This argument may not be generalized by substituting the idea or the word ''intermediate'' for ''peptide.'' An ''intermediate'' could be the activated amino acid.

Anfinsen and Steinberg ('51a, b; Steinberg and Anfinsen, '52) found unequal labeling of amino acids in ovalbumin and insulin and Vaughan and Anfinsen ('54) found it in ribonuclease. The inequality of labeling was greatest in the beginning and tended to grow less the longer the experiment. *In vivo* and *in vitro* experiments gave similar results (Steinberg and Anfinsen, '52).

Steinberg and Anfinsen ('52) were of the opinion that their results were explained best by the postulate of different-sized pools of preformed peptide intermediates. Labeled amino acids would first be incorporated into different-sized pools of the hitherto unlabeled peptides. The specific activity of the amino acid would then differ from peptide pool to peptide pool, and when these peptides combined to form the protein, the different segments corresponding to these peptides would contain the labeled amino acid at different specific activities.

The evidence against peptides playing such a role is as follows. This explanation calls for impossibly large amounts of preformed peptides (Borsook, '53). Repeated attempts to find such peptide intermediates, even in very small amounts, by use of labeled amino acids have failed.

In animal feeding experiments, peptides or partial hydrolyzates were less effective than amino acids or complete hydrolyzates (Silber and Porter, '49; Christensen, '50). Indeed, many peptides were excreted unused. In the tissue culture experiments of Gerarde *et al.* ('52), the greatest incorporation of labeled amino acids was obtained when the culture medium contained, instead of the embryo extract made up of proteins and peptides as well as amino acids, a mixture of nineteen amino acids.

In rabbit reticulocytes *in vitro,* the ratio of glycine incorporation into the heme and into the globin per residue of glycine over a wide range of rates of hemoglobin synthesis was one. This finding excludes the preexistence of any significant concentration of intermediates or precursors of heme or of globin more complex than free amino acids (J. Kruh and H. Borsook, unpublished observations).

Experiments on the formation of antibodies and of adaptive enzymes in microorganisms argue against more complex precursors than free amino acids in the formation of such specific proteins (Green and Anker, '54; Bulman and Campbell, '51; Ranney and London, '51; Gros *et al.,* '52; Rotman and Spiegelman, '54; Hogness *et al.,* '55; Halvorson and Spiegelman, '52; Spiegelman *et al.,* '55; Gale, '55).

The unequal labeling that led Anfinsen and his coworkers to envisage peptides as intermediates in protein synthesis has not been found by other workers under other experimental conditions. Thus Muir *et al.* ('52) found, after giving labeled amino acids to rats and rabbits, that the terminal and nonterminal valine of hemoglobin had the same specific activity. Velick and coworkers compared the specific activities of the same amino acid in three enzymes with different rates of turnover in muscle after injection of labeled amino acids to rabbits. Eight amino acids were examined. If there were any significant amounts of peptides, the specific activities of the same amino acid would be expected to differ in the three proteins. With all eight amino acids, the ratios of the specific activities in the three enzymes were the same. The conclusion was that the three enzymes were formed from the same amino acid pool and that a given amino acid in a protein is replaced at the same rate in all the positions in which it occurs (Simpson and Velick, '54; Heimberg and Velick, '54). Heimberg and Velick ('54) found that the main incorporation, i.e., synthesis of new protein, occurred within the first 2 hours after injection of the labeled amino acid.

Work and his collaborators earlier had made comparisons such as those of Velick and coworkers on casein and whey proteins; and their findings were the same (Campbell and Work, '52). Later, Askonas *et al.* ('54a, b) isolated peptides from partially hydrolyzed casein and compared the specific activities of a number of labeled amino acids common to them. They found that the same amino acid had the same specific activity in all the peptides, except the valine in one peptide. They concluded that casein is synthesized from free amino acids and that no peptides take part as such. Barry's findings ('52) were similar and he came to the same conclusion.

In experiments in this laboratory on hemoglobin synthesis *in vitro* in rabbit reticulocytes, a value of one was found for the molar ratio of incorporation of lysine and histidine per residue of amino acid in globin. This confirmed the conclusion drawn from the rates of glycine incorporation into heme and globin that there are no significant amounts of complex precursors (peptides) of globin. Muir *et al.* ('52) had come to the same conclusion earlier from their *in vivo* experiments.

If peptides participate in protein synthesis, their existence must be very transient and the amount present at any time must be very small.

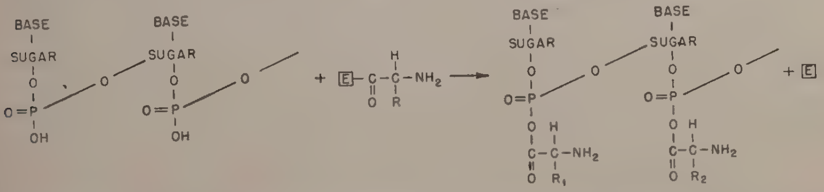
There can be no question of the unequal labeling observed by Anfinsen and coworkers, nor of the observations where unequal labeling was not found. But the explanation must be other than the presence of significant amounts of peptide intermediates. A possible explanation will be given.

RNA, THE PATTERN-FORMING TEMPLATE

The action of ATP was assigned to activation of the carboxyl groups of free amino acids by a special activating enzyme. Some have suggested that the activating enzyme is the template itself. Dounce ('52) has been the most explicit proponent of this view. In his opinion, ATP contributes the necessary energy by means of a phosphotransferase that transfers its terminal phosphate to the phosphate of nucleic acid; the net result is transfer of a pyrophosphate linkage from ATP to nucleic acid. The amino groups of amino acids then react to form amino-phosphate compounds on the nucleic acid, and for each amino group so combined the phosphate that came originally from the ATP is displaced and appears an inorganic phosphate. Another enzyme links the free carboxyl group of the adjacent amino acid to the phosphate-bound amino group to form a peptide linkage and the original phosphate of the nucleic acid. Diagrammatically, it is shown in figure 1.

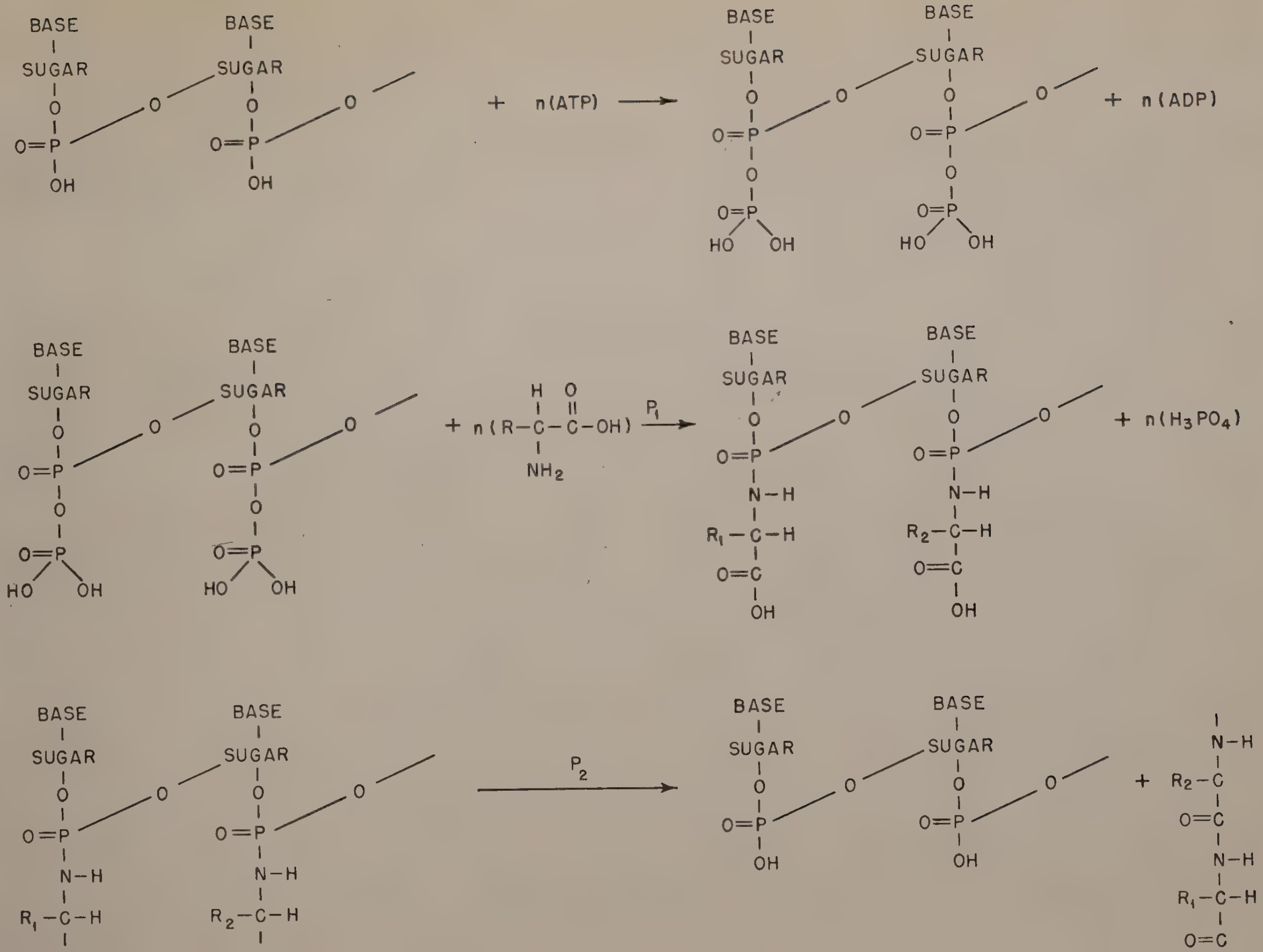
One class of enzymes, P_1, mediates attachment of the amino acids to the nucleic acid; another, P_2, effects the formation of the peptide bonds and the concomitant liberation of the peptide chain from the template.

In the hypothesis proposed by the author, the amino acids are attached to the phosphate of nucleic acid by their carboxyl groups. The scheme envisaged is as follows:



Then, as in Dounce's scheme, another enzyme forms the peptide bonds, thereby removing the amino acids involved from the nucleic acid template.

In the preceding scheme, some mechanism for transporting the activated amino acids to the template is called for. It might


BASE
SUGAR
+ n(ATP)
+ n(ADP)
+ n(R−C−C−OH) H O NH₂
P₁
+ n(H₃PO₄)
P₂
N−H
R₁−C−H
R₂−C−H
O=C
OH
HO

be the amino acid attached to the activating enzyme, or some smaller molecular weight carrier, a coenzyme, or nucleotide. Whatever it may be, the energy of activation is largely retained in the transfer from the activating enzyme to the template.

First, the basic notion that nucleic acid, and especially RNA, is so directly involved in protein synthesis needs to be examined.

More than a decade ago Brachet ('47a, b) and Caspersson (Caspersson and Brandt, '41; Caspersson *et al.*, '41; Caspersson and Schultz, '38; Caspersson and Thorell, '41) called attention to a mass of circumstantial evidence and adduced indirect experimental evidence implicating nucleic acids in protein synthesis. This evidence has been added to; for example, among the various cell fractions it is the particles with the highest concentration of RNA (Schneider, '47), i.e., the microsomes, which have the fastest incorporation of amino acids into their proteins (Borsook *et al.*, '50a; Hultin, '50; Keller, '51; Zamecnik and Keller, '54; Keller *et al.*, '54). Similarly, in the course of the development of reticulocytosis in rabbits, there is a thirty- to fortyfold increase in RNA and a roughly parallel increase in the ability to synthesize hemoglobin (and other proteins); the increase in DNA is only two- to threefold (Holloway and Ripley, '52; Koritz and Chantrenne, '54). Caldwell *et al.* ('50; Caldwell and Hinshelwood, '50) found in *Bacterium lactis aerogenes* that the RNA content of the cell was approximately proportional to the rate at which the cell had actually been growing, i.e., to the rate, *inter alia,* of protein synthesis. They suggested that in the synthesis of protein, the nucleic acid determines the amino acid pattern and, conversely, the amino acid sequence of the protein determines the specific configuration of the nucleic acid; this picture was accepted by Dounce ('52). Jeener and Jeener ('52) concluded from observations on experimentally induced variations in nuclear and cytoplasmic volumes of

Thermobacterium acidophilus that protein synthesis was quantitatively related to that of RNA, and that its dependence on the presence of DNA is much more remote and indirect. In yeast, the adaptive formation of galactozymase is inhibited by ultraviolet light; the action spectrum of the inhibition is that of nucleic acid and markedly different from that of protein (Swenson, '50).

Direct experimental evidence has now been found implicating RNA in protein synthesis. Gale and Folkes ('53a) showed that protein synthesis in the particles of a cell-free preparation of *S. aureus* was abolished by treatment with ribonuclease. Lester ('33) and Beljanski ('54) found the same in a cell-free preparation of *Micrococcus leisodeikticus.* The incorporation of labeled amino acids by onion root tips (Brachet, '54) and by rat liver microsomes (Zamecnik and Keller, '54) is inhibited by ribonuclease.

The dependence of protein synthesis on DNA is less direct than it is on RNA. Amino acid incorporation and protein synthesis can be demonstrated *in vitro* in the non-nucleated fractions of mammalian cells (Borsook *et al.,* '50a, b; Hultin, '50; Keller, '51; Zamecnik and Keller, '54; Keller *et al.,* '54; Allfrey *et al.,* '53; Mirskey *et al.,* '54; Brachet and Chantrenne, '51; Loftfield, '54). Non-nucleated fragments of protozoa incorporate labeled amino acids into their proteins (Brachet and Chantrenne, '51). X irradiation (Hevesy, '49; Brandt *et al.,* '51; Abrams, '51; Baron *et al.,* '53), which inhibits synthesis of DNA, does not inhibit RNA and protein synthesis to the same extent, or it may not inhibit the latter at all. At doses of ultraviolet radiation that inhibit DNA synthesis, RNA and protein synthesis continue (Spiegelman *et al.,* '55).

Sulfur mustard stops growth in *Escherichia coli* (i.e., DNA synthesis) but not adaptive enzyme formation (Sher and Mallette, '54; Pardee, '54). A mutant of *E. coli* that requires thymine for DNA synthesis, in the absence of thymine and unable to multiply, could produce adaptive enzymes (Cohen and Barner, '54).

RELATION BETWEEN RNA SYNTHESIS AND PROTEIN SYNTHESIS

The mere presence of some RNA is not sufficient for protein synthesis (Holloway and Ripley, '52; Brachet, '54; Cohen and Barner, '54). Mature rabbit erythrocytes contain about 40 mg% of RNA (Holloway and Ripley, '52) and yet they cannot synthesize protein. On the other hand, there need be no net increase in RNA for extensive synthesis of protein (Jeener, '52); but in every case where there is rapid protein synthesis, turnover (i.e., synthesis of RNA) is observed (Gale and Folkes, '54; Gale, '55; Caldwell *et al.,* '50; Caldwell and Hinshelwood, '50; Gale and Folkes, '53b; Reddi, '55). A strong positive correlation was observed between the two processes in a number of different microorganisms and in the synthesis of a variety of different proteins. Conversely, inhibition of RNA synthesis inhibited protein synthesis. 5-Hydroxyuridine inhibited RNA synthesis in *E. coli,* it did not stop growth, but it inhibited synthesis of β-galactosidase. After depletion of pyrimidines in pyrimidineless mutants of yeast, the synthesis of induced enzymes was greatly retarded; when purines and pyrimidines were added, there was a prompt restoration of enzyme formation (Spiegelman *et al.,* '55). Similar results were obtained by Pardee ('54).

Gale and Folkes observed that the addition of purines and pyrimidines, RNA, or DNA increased the rate of synthesis of protein, including a number of enzymes in intact *S. aureus* and in the cell-free particles (Gale and Folkes, '53b, d, '54; Gale, '55). The effect on rates of synthesis varied with different amino acids. In general, stimulation of synthesis of either RNA or protein stimulated the synthesis of the other.

From findings such as the foregoing, Spiegelman *et al.* ('55) and Pardee ('54) suggested that, for the synthesis of protein, new RNA must be synthesized concomitantly.

Stimulation of nucleic acid synthesis and protein synthesis do not always go together. Chloromycetin stimulates nucleic acid synthesis in *S. aureus* and inhibits protein synthesis

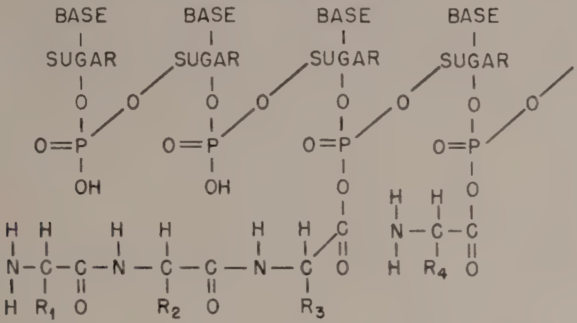
(Gale and Folkes, '53c). In the vitamin E-deficient rat, the turnover of both DNA and RNA in the intestine, liver, and muscle is faster than normal without any significant difference in protein turnover (Dinning, '55).

In rabbit reticulocytes, there is rapid protein synthesis and at the same time, a rapid net decrease in RNA (Kruh and Borsook, '55). These cells, under optimal conditions, synthesize hemoblogin at a rate of about 300 mg in 4 hours per 100 ml of cells. On the assumption that the molecular weight of RNA is about five times that of hemoglobin if 1 molecule of RNA must be synthesized per molecule of hemoglobin synthesized, then 1500 mg of RNA would have to be synthesized in 4 hours, or twice the total RNA originally present, at a time when the RNA in these cells is rapidly decreasing. There is some synthesis of RNA, the rate could not be measured, but it appears to be only a very small fraction of 1500 mg in 4 hours (Kruh and Borsook, '55).

An explanation that suggests itself is that only part of the RNA is active or native; the rest is inactive or denatured. Only native (active) RNA can serve as a template for protein synthesis. The RNA is continually being denatured and hence, for active protein synthesis, new RNA must continually be synthesized, but not necessarily on an equimolar basis. Hershey ('53) in an analysis of the effects of bacteriophage on RNA and DNA metabolism in *E. coli,* came to the conclusion that a distinction must be made between the metabolically active RNA and the bulk of the RNA in the cells before infection.

SOME CONSEQUENCES OF THE DIFFERENCE IN STRUCTURE OF RNA AND OF PROTEIN

A present view of the structure of RNA is that it is a rather shallow spiral. The pitch of the spiral is much less than that of proteins in their helical form. Consequently, protein cannot take its normal form while attached to the template. There is less strain to the protein, and to these explanations, if it is assumed that, on the template, the carboxyl groups are attached and the amino groups are free. In other words, there are no peptide bonds; as soon as peptide bonds are formed that much of the peptide chain uncoils, and dangles, as it were, as in the following diagram.



For different reasons, a picture of incomplete proteins dangling from the template has been proposed by Dalgliesh ('53). In his picture, many peptide chains at different stages of growth may be attached to the template at the same time.

The specificity of position of the amino acids on the template is determined by the topography of the space created by the purine and pyrimidine bases. Because the spiral is a shallow one, the space for any one amino acid is made, not by the bases that immediately follow one another in the nucleotide chain, but by bases that are possibly as much as ten nucleotides distant.

Electron microscope studies indicate that the RNA of the cytoplasm is a reticulum of vesicles and canaliculi (Palade and Porter, '52; Howatson and Ham, '55). Presumably, the microsomes, which are the most active protein-synthesizing material in the cell, are derived from this reticulum.

It is easier, with a reticulated RNA template, to envisage the formation of long peptide chains with disulfides cross links and reversals of the helix. At any rate, there is direct evidence now for extended RNA, which makes it easier to envisage its serving as a template.

THE RELATION OF THE LEVEL AND THE QUALITY OF NITROGEN INTAKE TO THE RATE OF PROTEIN SYNTHESIS

Obviously, no protein will be synthesized unless all the spaces on the template are filled by amino acids. The rate of protein synthesis will be dependent on the rate at which the template is filled. This rate will depend on the rate of activation of amino acids which in turn will depend on the rate of production of ATP and on the concentration of free amino acids. Table 2 shows the effect of amino acid composition on the rate of protein synthesis in rabbit reticulocytes. It may be assumed that in the cells of the blank there were some amino acids, and these were replenished at a slow rate by the breakdown of reticulocyte protein. The results show that a limitation of one amino acid limits the rate of the whole process. This limitation need not be an absolute deficiency; a suboptimal concentration will be limiting. It is significant that the concentration of amino acids in rabbit plasma was far from optimal; the rate could be more than doubled simply by enrichment with histidine, leucine, phenylalanine, and valine (Borsook *et al.*, '52).

TABLE 2

Effect of amino acid composition on the rate of protein synthesis in vitro in rabbit reticulocytes

(Results expressed as percentage of Blank without added amino acids)

AMINO ACID COMPOSITION	PERCENTAGE OF BLANK IN NO. OF HOURS INDICATED			
	1	2	3	4
Complete	447	450	489	475
Complete without histidine	94	87	94	96
Complete without valine	185	170	181	187
Complete without phenylalanine	176	183	181	200
Complete without serine	215	227	220	228
Complete without lysine	235	229	237	222
Complete without tryptophan	257	267	264	285
Complete without tyrosine	262	280	295	297
Complete without glutamine	304	339	353	366

The major effects of a protein meal on the nitrogen balance, as on the specific dynamic action, are within the first few hours after it is eaten, i.e., during the time that the amino acid level in the blood and tissues is increased. This has been observed with labeled amino acids (Borsook *et al.,* '50a; Heimberg and Velick, '54). Similarly, it has been found in feeding experiments that an indispensable amino acid is ineffective for growth, for recovery from protein depletion, or for maintenance, unless it is fed or injected within a few hours of other necessary amino acids (Cannon *et al.*, '47; Geiger, '47, '48b, '50b, '51; Geiger *et al.,* '49; Harte *et al.,* '48; Henderson and Harris, '49; Schaeffer and Geiger, '47; Yeshoda and Damodaran, '47). So long as all the necessary free amino acids are in the tissues, which they always are, there is always some protein synthesis. A single, labeled amino acid, whether dispensable or indispensable, is extensively incorporated into an animal's protein within a few minutes (Borsook *et al.,* '50a), whether the animal is in a normal nutritional state or is fasting (Tarver and Schmidt, '42). It is a question of more or less synthesis. The critical parameter is the concentration of the amino acids and the completeness of the mixture. In an incomplete amino acid mixture, the rate of protein synthesis will be slower the more incomplete it is and, accordingly, the greater will be the negative nitrogen balance. The difference in the biological quality of proteins or of amino acid mixtures resides in how high will be the concentration of all the needed amino acids in the tissues during the first few hours after ingestion. If a protein is short in one indispensable amino acid, obviously more of it must be eaten to maintain such a level of protein synthesis that nitrogen balance (or growth) is maintained. The different amino acid mixtures in table 2 could be considered as different proteins. Tables 3, 4, and 5 show that intermediate effects can be obtained by addition of graded amounts of any one of the limiting amino acids.

TABLE 3

Effect of partial histidine deficiency on rate of protein synthesis in vitro in rabbit reticulocytes

(Results expressed as percentage of that with maximum histidine added)

HISTIDINE ADDED WITH OTHERWISE COMPLETE AMINO ACID MIXTURE		RATE IN SUCCESSIVE HOURS			
		0–1	1–2	2–3	3–4
µg/ml	M ($\times 10^{-4}$)	%	%	%	%
0	0	23	18	27	23
9.25	0.575	81	30	33	24
18.5	1.15	104	77	39	25
27.75	1.72	100	98	93	47
37.0	2.30	100	98	88	80
46.25	2.87	100	97	98	102
74.0	5.75	100	100	100	100

TABLE 4

Effect of partial phenylalanine deficiency on rate of protein synthesis in vitro in rabbit reticulocytes

(Results expressed as percentage of that with maximum phenylalanine added)

PHENYLALANINE ADDED WITH OTHERWISE COMPLETE AMINO ACID MIXTURE		RATE IN SUCCESSIVE HOURS			
		0–1	1–2	2–3	3–4
µg/ml	M ($\times 10^{-4}$)	%	%	%	%
0	0	35	37	39	38
6.5	0.39	88	66	62	61
13.0	0.78	90	90	87	80
19.5	1.18	92	93	105	98
26.0	1.57	93	92	100	102
32.5	1.96	94	93	102	101
65.0	3.93	100	100	100	100

TABLE 5

Effect of partial valine deficiency on rate of protein synthesis in vitro in rabbit reticulocytes

(Results expressed as percentage of that with maximum valine added)

VALINE ADDED WITH OTHERWISE COMPLETE AMINO ACID MIXTURE		RATE IN SUCCESSIVE HOURS			
		0–1	1–2	2–3	3–4
µg/ml	M ($\times 10^{-4}$)	%	%	%	%
0	0	. .	29	. .	32
9.25	0.78	81	61	50	53
18.5	1.57	98	85	71	73
27.75	2.36	100	97	84	86
37.0	3.15	95	99	92	98
46.25	3.94	95	99	94	97
92.5	7.89	100	100	100	100

SLOWNESS OF PROTEIN SYNTHESIS

Protein synthesis is slow as compared with other enzymic reactions. For purposes of comparison, the whole process may be considered as if it were mediated by a single enzyme. If it is assumed that the molecular weight of the protein and of the hypothetical enzyme are the same, then the turnover number of the enzyme is

$$\frac{\text{Weight of protein synthesized per minute}}{\text{weight of enzyme}}.$$

In reticulocytes, the maximum rate of hemoglobin synthesis observed was 6.4 mg/g of total protein per hour. If it is assumed that the enzyme was of the order of 1% of the total protein, then the rate was 640 mg/g of enzyme per hour or 10.6 mg/g of enzyme per minute, or a turnover number of approximately 10^{-2}. If the turnover number is taken as the number of amino acid residues built into protein, with approximately 580 amino acid residues per mole of hemoglobin (Schroeder *et al.*, '50), the turnover number per residue is 5.8. The turnover number of ordinary enzyme reactions is 100–3,000,000. Even in bacteria, during the logarithmic phase of their growth, when protein synthesis is 1000 times as fast as the fastest rates in mammalian tissues, the turnover number per amino acid residue is of the order of 5000, which is in the slow range of ordinary enzyme rates.

INHIBITION ON THE TEMPLATE

A priori, it is to be expected that an inhibitory amino acid analog would block its natural counterpart on the template and thereby block the utilization of all the other unrelated amino acids for protein synthesis. Halvorson and Spiegelman ('52) demonstrated this effect in yeast with inhibitory analogs of leucine, methionine, and phenylalanine; only the natural counterpart could relieve the inhibition.

In at least one case the inhibitory analog is incorporated, i.e., an abnormal protein is formed. This is the case with ethionine in the rat (Levine and Tarver, '50, '51). As was to be expected, it was incorporated more slowly; its "half life" in the tissue proteins was shorter than that of its natural counterpart, methionine, and it slowed down the incorporation of other amino acids into protein.

Peptides accumulate in microorganisms when protein synthesis is deranged as in *S. aureus* with glucose and an incomplete amino acid mixture (Gale, '51, '53, '55), or in the presence of penicillin (Hotchkiss, '50; Park, '52a, b, c). Among these are the peptides Park has isolated and found to be attached to uridine nucleotides (Hotchkiss, '50). These peptides may be incompletely formed protein which comes off the template as a result of the derangement by an incomplete amino acid mixture or by the antibiotic. Chloromycetin and aureomycin have a small, if any, inhibiting effect on the formation of these peptides (Gale, '53). The findings, as a whole, point to interference on the template as the locus of action of chloromycetin and penicillin. Aureomycin, which at 400 μg/ml uncouples oxidative phosphorylation (Loomis, '50), would at this concentration inhibit the activation process; the data of Gale and Paine ('51) suggest that this is the case; they found that peptide formation is inhibited by a lower concentration of aureomycin than of chloromycetin. But both antibiotics inhibit protein synthesis at much lower concentrations, e.g., 20 μg/ml (Gale, '55; Levine and Tarver, '50, '51; Loomis, '50). At this concentration, their inhibitory action is at the template. Such peptides would hardly be expected except where protein synthesis is very rapid, as in microorganisms.

AMINO ACID EXCHANGE IN PROTEINS

Operationally, amino acid exchange means one of two findings—unequal labeling of a given amino acid at different loci in the protein molecule, or incorporation of an amino acid into a protein under conditions where synthesis of protein *de novo* from amino acids is excluded.

Reference has been made to the unequal labeling observed by Anfinsen and his coworkers. The inequality of labeling is greatest at the begining of an experiment, it grows progressively less with time, and this variation in the inequality of the labeling is not caused by changes in the specific activity of the free amino acid.

An explanation that brings these observations into line with a number of others is as follows. Protein synthesis is a slow reaction. For a finite time, certain loci on the template are unoccupied and the protein cannot be released to the medium until all the loci are occupied. During this time the amino acids bound to the template but not to one another can exchange with other activated amino acids coming from the free amino acid pool. At the beginning of an experiment, the empty spaces on the template will be filled by labeled amino acid, and also the labeled amino acid may exchange with unlabeled amino acid on the template, at different rates at different loci. Both processes would give unequal labeling when, after a time, the template is fully occupied and the protein is released from the template. From then on, the same amino acid will be equally labeled at all loci in the protein molecule.

The second kind of amino acid exchange, incorporation of an amino acid into a protein under conditions where synthesis of protein *de novo* from amino acids is excluded, has been observed experimentally. Excluded here is the special case of lysine incorporation into a guinea pig liver protein (Borsook, '50, '52, '53). Reference is made to experiments such as those described by Gale and Folkes ('53e, '54; Gale, '55) where washed *S. aureus* supplied with a single amino acid and a source of energy, incorporated the amino acid into its protein, and there was no increase in cellular protein. The incorporation was inhibited by inhibitors that prevent ATP formation, i.e., amino acid activation. That an actual transfer occurs was proved by beginning with labeled protein in the bacteria and finding labeled amino acid, after the exchange, free in the medium.

This kind of exchange appears to be the most reasonable explanation for the observations of Rabinovitz *et al.* ('54) that leucine, lysine, and valine were incorporated into ascites carcinoma cells in the presence of a concentration of a phenylalanine analog that blocked completely the incorporation of phenylalanine.

In the tissue-culture experiments of Francis and Winnick ('53), the findings on the effects of amino acid analog inhibitors suggest (they are not proof) that the incorporation of free amino acids can be inhibited while proteins (or protein fragments) remain available for limited protein synthesis or for turnover processes.

It has been found in rats whose proteins were labeled with either glycine or alanine that the rate of disappearance of the labeled amino acid from the animal's proteins was greater when glycine or alanine, respectively, was continuously infused. Alanine was ''washed out'' best by alanine, glycine by glycine (N. Bucker, personal communication from R. B. Loftfield).

This second mode of amino acid exchange is readily accounted for if it is postulated that preformed protein can be, and is, reimposed on the template. If the terminal carboxyl group is to be attached, it would require activation, i.e., utilization of ATP. A part of the protein might be attached at the locus of an internal peptide bond, via cleavage of the protein, and with the energy of the peptide bond conserved by attachment of the newly formed carboxyl group to the enzyme and thence to the template. To the extent that the protein or peptide is attached to the template, all its peptide bonds are broken; the rest dangles from the template. Exchange would occur only in those parts that are attached to the template. Energy is required for the exchange because free amino acids of the pool must be activated before they can exchange.

In general, it would be expected from considerations of the geometry and probability that reimposition of a protein or peptide on the template would be a much slower process than protein synthesis *de novo* from amino acids and that there is a competition for the template between activated amino acids and preformed protein. The rate of turnover of a preformed protein would be governed by the amount of enzyme available for attaching it to the template and by the competition from new protein synthesis from amino acids.

The process envisaged is:

Protein $\rightleftharpoons$ (template) amino acid $\rightleftharpoons$ activated amino acid $\rightleftharpoons$ free amino acid

By a scheme such as this, nitrogen balance in animals is maintained. An increase in free amino acids increases synthesis of protein; increase of any one kind of protein (so long as it is not, like hemoglobin, excluded from metabolism) increases the rate of breakdown of that protein. Hence the close correlation in different tissues between over-all turnover as measured over periods of days by N^{15} labeling and rates of incorporation *in vitro* or *in vivo* measured with C^{14} labeling in an hour or two (Borsook, '50, '52).

PROTEIN TURNOVER

The meaning of the term "turnover," as applied to proteins, should be reexamined. Originally, it meant the exchange of labeled for unlabeled amino acids in proteins of animals. Since all amino acids are exchanged, most of, if not all, the peptide bonds must be broken and reconstituted. The rate of this exchange in the animal as a whole is greater than was envisaged in Folin's theory of endogenous and exogenous metabolism. This then led to the concept of the dynamic state of proteins, that all the proteins are continually being broken down and rebuilt. Enzymes, hormones, and structural proteins are all involved in the process. There is no operational utility in distinguishing between structural and other proteins (Borsook and Keighley, '35; Schoenheimer, '42; Borsook, '50, '52).

Schoenheimer in his earliest papers pointed out the great differences in rate of turnover of the proteins in different tissues. Later, evidence was adduced that most of the turnover, as measured by the nitrogen in the urine, must be attributed to the proteins of the viscera (Borsook, '50, '52).

The rate of turnover, in an animal in nitrogen balance, must be proportional to the nitrogen intake. Only about 50%

of the excreted nitrogen on any one day comes from the food eaten on that day; the rest must come from tissue protein and there must, then, be an equal amount of protein resynthesis. This inference was proved directly by Solomon and Tarver ('52), who found that a high protein intake accelerated loss of methionine-S^{35} from the proteins of plasma, liver, and kidney. The findings on the "washing out" of labeled amino acids from proteins, just referred to, suggest that the term "turnover" would be used more accurately if it were always modified by reference to a specific amino acid, because the turnover rate of a given amino acid in a protein is evidently affected by the concentration of that amino acid free in the pool.

The same dependence of the rate of protein breakdown (and resynthesis) on the nitrogen supply has been demonstrated by Podolsky ('53) in *E. coli.* He found that increase in amino acids in the medium increased both the rate of growth, i.e., protein synthesis, and also the rate of protein breakdown. Of course synthesis was increased more than breakdown. The results with phosphorus are different; once taken up, apparently none of it is returned to the medium during subsequent growth. This one-way flow of phosphorus has been observed in other microorganisms (Labaw *et al.,* '50; Kaudewitz, '53; Manson, '53). It appears not to be the case in mammalian tissues, e.g., liver. In that organ, the RNA declines sharply in 2 days' starvation and increases again after feeding; the DNA remains unchanged (Davidson, '47; Brachet *et al.,* '46).

The normal breakdown, like the incorporation of amino acids, in animal tissues is dependent on the supply of energy. Dinitrophenol inhibits excorporation as well as incorporation in liver slices (Simpson, '51). The same occurs in microorganisms. The disappearance of an induced enzyme from yeast after removal of the inducer can be prevented by agents that inhibit its formation, e.g., azide and dinitrophenol, which either inhibit respiration or uncouple phosphorylation from oxidation (Sussman and Spiegelman, '50; Jebb *et al.,* '50; Spiegelman and Reiner, '47). As in animals, some of the proteins in yeast are more labile than others (Halvorson and Spiegelman, '53).

The whole concept of the dynamic state of proteins has been questioned by Hogness *et al.* ('55) on the basis of their observations of the great stability of induced β-galactosidase in *E. coli.* Their observation has been confirmed by Rickenberg *et al.* ('53). Hogness *et al.* assert flatly that the proteins in *E. coli* are static. The measurements of Cowie *et al.* ('50) with S^{35}, the same tracer as used by Hogness *et al.,* indicate that exchange of protein-bound sulfur with the environmental sulfur during growth is less than 0.5% per hour. This rate corresponds to the fastest observed in mammalian tissues, but is only one-thousandth as rapid as the synthesis of protein in *E. coli* during its logarithmic phase of growth, and is within the experimental error of the measurements of Hogness *et al.* ('55) and Rotman and Spiegelman ('54). Not all the proteins in *E. coli* are as stable as β-galactosidase; the observations of Podolsky ('53) indicate that there are less-stable proteins in this organism, and this point was demonstrated directly with induced lysine decarboxylase, which disappears in one and one-half generations after removal of the inducer (Sher and Malette, '54).

There can be no disagreement with Hogness and coworkers that turnover rates are a kind of measure of the dynamic state of a tissue. But in any one tissue, some proteins are broken down and are rebuilt faster than others, and to this extent one may refer to the dynamic state of those molecules; the differences in their dynamic state can be measured. In liver, different cell fractions and different enzymes within them have different rates of catabolism during protein starvation. Indeed, the xanthine oxidase of liver has been found to be a sensitive index of the availablity and biological value of proteins and amino acid mixtures (Borsook, '50, '52).

MODE OF FORMATION OF NEW TYPES OF PROTEINS: ANTIBODIES AND ADAPTIVE ENZYMES

In the picture of the mechanism of protein synthesis outlined here, antibody formation would be as follows: The globulin (or whatever the protein precursor is) amino acid pattern is established on the nucleic acid template; it is much more extended than in the final helical structure. In the absence of an antigen, in coming off the template, it takes its stable, nonantigenic helical structure. In the presence of an antigen, as the protein comes off the template, it combines loosely with the antigen and the result is a different, stable helical structure that is antigenic. The dissociation of the antigen from the antibody would then be effected by changes in salt concentration or *p*H in the neighborhood of the template as the protein coils away from it.

The formation of adaptive enzymes would, in principle, be the same as the formation of antibodies. It differs only in that the deforming principle is of smaller molecular weight; it appears that antigens must have a molecular weight of at least 12,000, whereas a simple sugar, even formate, can function as an inducer of adaptive enzymes.

In this view there is not a special template for each antibody or for each adaptively formed enzyme. A certain kind of protein, since there is a template for it, will form antibodies. From this one protein on its template, many different antibodies can be formed, by differences in coiling of the peptide chain as it comes off the template. Analogously, one kind of protein may form a number of different adaptive enzymes and, according to the degree of stability of the new protein, it may persist or disappear after removal of the inducer. This picture is in accord with such findings as that the induction of a new enzyme in a cell can result in the disappearance of an existing enzyme and suppress the formation of others; and this suppression is reduced greatly when the exogenous nitrogen supply is abundant (Spiegelman and Reiner, '47). From this view, it would be expected that new enzymes could be formed under conditions when there is no net increase in protein. This has been observed even with β-galactosidase in *E. coli* (Bonner, '55). The picture proposed is in accord with Dubnoff's findings ('55) that a protein, potentially capable of forming an adaptive enzyme, in the absence of the adapting substrate is inactivated, but can be regenerated, without inducer being present, by glucose 6-phosphate, glutathione, and vitamin B_{12}.

REGULATION OF PROTEIN AND NUCLEIC ACID SYNTHESES

So long as a protein is fully attached to a template, that template is inactive and so is the protein. If the two remain attached permanently, it is analogous to mutual precipitation. One may ask "Is this not the reason that virus and phage divert the whole economy of the cell toward the production of the virus or phage?"

In an analogous manner, the rate of formation of the heme may regulate the rate of synthesis of globin. In the reticulocytes, i.e., in the late stages of maturation of the red cell, observations indicate that the rates of heme and globin synthesis are practically equal.[4] In more immature red cells (in the bone marrow — Muir *et al,* '52), heme synthesis appears to be faster than globin synthesis. The synthesis of heme and of protein can be dislocated by radiation or by fasting (Salomon *et al.*, '49; Richmond *et al.*, '51); the synthesis of heme is depressed less than that of globin. But even under such exigencies, heme synthesis is less than double that of globin. It is remarkable that the two processes do not run very differently. *A priori,* there is no chemical reason why the two rates should be nearly the same; there must be regulation. Such regulation is deducible from the picture of protein synthesis outlined without introducing any additional *ad hoc* process.

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[4] The same result is obtained from the *in vivo* observations of Altman *et al.* ('48), if their data are recalculated to take into account the dilution of the labeled glycine in the reticulocytes, and a later and more reliable figure than theirs is used for the glycine composition of globin.

GENERAL DISCUSSION

HAUROWITZ[5]: I think that I was one of those who proposed incorporation of free amino acids, not of peptides. It has been objected by Synge that incorporation of amino acids at a template would involve a multimolecular reaction. This objection is not valid because a zipper-like reaction can be imagined in which one amino acid after the other would be incorporated. The intermediates containing two, three, four, or more amino acids may be considered as peptides. In contrast to free peptides, however, they are bound to the template. The maximum time required for the synthesis of a protein molecule can be calculated from the time of antibody molecules formed per antigen molecule injected. This time is of the order of 1 second per protein molecule formed.

STEINBERG[6]: I should like to make a few clarifying comments on Dr. Borsook's reference to the work we have been doing in Dr. Anfinsen's Laboratory.

Our data on nonuniform labeling of proteins synthesized *in vitro* have been amplified and extended. Three crystalline proteins (ovalbumin, insulin, and ribonuclease) have now been studied and nonuniformity of labeling was demonstrated in all. Although the results allow no simple interpretation, they do at least rule out synthesis by a simultaneous condensation of free amino acids. Some of the several explanations proposed were: (1) Formation of intermediate compounds: I should like to emphasize that we said *free* or *bound* intermediates. On a kinetic basis, there is no difference in the end result whether the intermediates are free or bound as long as they are kinetically distinct. (2) Reversible reactions: One or more reversible reactions between the free amino acid level and the protein level, with or without intermediate compounds, could result in nonuniform labeling if the rates were different for different residues. (3) Changing precursor specific activity: If the time for synthesis of the protein were long and the precursor changed in specific activity during the synthesis of one macromolecule, nonuniform labeling would result.

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[5] Felix Haurowitz, Indiana University.

[6] Daniel Steinberg, National Heart Institute.

We have made some calculations also to estimate the size of intermediate pools needed for an explanation of the findings. Using Christensen's data on the amino acid content of oviduct and our measurements of ovalbumin turnover, we estimate that an intermediate pool of only 2% of the molar concentration of the free amino acid pool would give nonuniform labeling results similar to ours.

We have no information about the nature of the intermediates postulated on the basis of kinetic studies. However, Dr. Hoagland in Dr. Zamecnik's laboratory has elegantly demonstrated what may represent the first intermediate in protein synthesis, namely, an amino acid-adenylic acid-enzyme complex. If it develops that there is more than one form of the activated complex for a given amino acid species, an explicit basis for nonuniform labeling will be at hand.

The apparent discrepancy between the results of Work and his collaborators and our results may be explained on the basis of a kinetic analysis. The same pattern of synthetic reactions can lead to uniform or nonuniform labeling depending on the rate of protein synthesis, the contribution of reversible reactions and the availability of precursor material in the system studied.

GUILD[7]: Dr. Borsook suggests that RNA acts as a template for protein synthesis. We have discussed this question at Yale and with Henry Quastler of Illinois and one calculation of the length of time allowed for synthesis of one protein molecule may be worth while. It is simply this:

When a reasonable molecular weight is assumed for RNA, from the chemical analyses there appear to be 10^4–10^5 RNA molecules per bacterial cell. Furthermore, there are 10^6–10^7 protein molecules per bacterium. In log phase, the generation time is of the order of 10^3 seconds. Therefore, 10^3–10^4 protein molecules must be synthesized per second. If RNA is the template, there are 10^4–10^5 of them, and any one template has of the order of 10 seconds to manufacture one protein molecule. This number is probably correct within a factor of 10.

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[7] W. R. Guild, Yale University.

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MECHANISM OF INCORPORATION OF LABELED AMINO ACIDS INTO PROTEIN[1]

P. C. ZAMECNIK, E. B. KELLER, J. W. LITTLEFIELD,
M. B. HOAGLAND,[2] AND R. B. LOFTFIELD
*Medical Laboratories of the Collis P. Huntington Memorial Hospital
of Harvard University, at the Massachusetts
General Hospital, Boston*

FIVE FIGURES

In this supplement, a general review of studies on the mechanism of protein synthesis is presented by H. Borsook. The present discussion may therefore, with less embarrassment, be confined to a presentation of recent experimental findings from a single laboratory. These results are concerned principally with cell-free preparations from rat livers, and will be introduced under four main headings.

In 1941 Lipmann prophesied that phosphate-bond energy drove the process of protein synthesis. The availability of C^{14} in the postwar years made it possible to devise an experimental test of this suggestion. Using dinitrophenol (DNP), Frantz *et al.* ('48) found a parallelism between the inhibition of phosphorylation and of amino acid incorporation, in liver slices. Siekevitz ('52; Siekevitz and Zamecnik, '51) extended this theme to a cell-free system, demonstrating a relation between oxidative phosphorylation and amino acid incorporation in a crude rat liver homogenate. Other evidence for the participation of phosphate-bond energy in the synthesis of protein and of peptidic bonds had also continued to accumulate (Speck, '47; Elliott, '48; Bloch, '49; Peterson and Green-

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berg, '52). At about this time, Bucher ('53) had found that C^{14}-acetate could be built into cholesterol in a cell-free liver homogenate, provided that gentle homogenization was employed. When this loose homogenization was adopted in the search for a more active cell-free incorporation system, and hexosediphosphate (HDP) was added as an energy donor, the incorporation rate was increased approximately tenfold (Zamecnik, '53). It was also possible to discard the mitochondrial fraction of the cell-free rat liver homogenate and to run the incorporation reaction anaerobically, HDP being used as an energy source. At this point (Zamecnik and Keller, '54), the incorporation system consisted of four components: (1) the labeled amino acid, (2) the energy donor, (3) the microsome fraction, and (4) the $105{,}000 \times g$ supernatant fraction. Substitution of phosphocreatine and adenosine triphosphate-creatine transphosphorylase for the HDP-glycolytic enzyme energy generator provided a further simplification of the system, but did not eliminate the necessity for component (4). It was possible, however, to dialyze out small molecular weight components from this $105{,}000 \times g$ supernatant, and in this way to demonstrate a requirement for added ATP.

It was therefore reasonable to consider that either the microsomes or the amino acids themselves were being activated by adenosine triphosphate (ATP).

AMINO ACID ACTIVATION

In order to distinguish between these possibilities, Hoagland ('55a) studied the rate of exchange of radioactive pyrophosphate (PP^{32}) into ATP in the microsome and $105{,}000 \times g$ supernatant fractions. In the latter fraction, it was found that the addition of a complete complement of L-amino acids enhanced the rate of exchange of PP^{32} into ATP. This observation provided the encouragement to try to trap carboxyl-activated amino acids, if such were being formed in this $105{,}000 \times g$ supernatant fraction in the presence of ATP. By the use of high concentrations ($1.2\,M$) of hydroxylamine, α-aminohydroxamic acids were found. It was necessary to use salt-free hydroxylamine in order to prevent a high salt concentration from inhibiting the enzymic reactions.

Interestingly enough, the extent of formation of hydroxamic acid was dependent on the number of separate amino acids added to the 105,000 × *g* dialyzed supernatant. This finding pointed to multiple, separate activating enzymes (or multiple separate activation sites) rather than activation of all amino acids at a single enzymic locus in the 105,000 × *g* protein (Hoagland, '55b; Hoagland *et al.*, '56). This latter fraction, containing soluble proteins in the liver cell, can be partially precipitated at *p*H 5.2. The amino acid-activating enzymic fraction precipitates at this *p*H, and can be redissolved, with several-fold enhancement of specific enzymic activity.

TABLE 1

Stoichiometry of hydroxamic acid, inorganic phosphate, and inorganic pyrophosphate formation

ADDITIONS	HYDROXAMIC ACID	PHOSPHATE	PYROPHOSPHATE
	μmoles	*μmoles*	*μmoles*
None[a]	0.1	1.3	0.3
Amino acids[b]	1.2	1.5	1.3

[a] 7 mg of *p*H 5.2 enzyme; 10 μmoles of K_4ATP, 1 μmole of $MgCl_2$, 1.2 μmoles of NH_2OH.

[b] 4 μmoles each of twelve L-amino acids.

1 ml, final volume, incubated at 37°C. for 60 minutes.

Under these conditions of a low Mg^{++}/ATP ratio, pyrophosphatase activity is inhibited and pyrophosphate can therefore be detected. This is not the case where the Mg^{++}/ATP ratio is around 0.5.

A correlation between number of moles of hydroxamic acid formed and pyrophosphate liberated in this reaction indicated a pyrophosphate splitting of ATP, with formation of an aminoacyl-AMP (adenosine monophosphate) intermediate (see table 1). Since amino acids do not cause a detectable splitting of ATP unless hydroxylamine is added, the aminoacyl-AMP compound is either bound to an enzyme or is free in such small concentrations that it is as yet unobserved.

THE PARTICIPATION OF GUANOSINE DIPHOSPHATE AND GUANOSINE TRIPHOSPHATE IN INCORPORATION

When the microsome fraction was centrifuged out of four times the usual volume of suspending medium, and the pH 5.2 precipitated protein fraction used in place of the entire 105,000 × g supernatant fraction, it was found that ATP could no longer activate an otherwise complete system. It was suspected that some other nucleotide might be involved. Dr. Rao Sanadi kindly furnished guanosine diphosphate (GDP) prior to its commercial availability. It was found, under circumstances in which the protein fractions of the system were freed of the great bulk of accompanying nucleotides, that GDP or GTP (guanosine triphosphate) played a special activating role independent of ATP (Keller and Zamecnik, '55, '56). In order to demonstrate this separate GDP effect, it was necessary to use ATP of high purity.

It is not clear how the GDP operates. There is a choice of considering that it serves as an intermediate aminoacyl transfer compound, operating between the ATP-amino acid activating system and the microsomes, or that a particular amino acid is activated by GTP rather than by ATP. This latter possibility is less likely, since the activation of amino acids has thus far been found to be ATP specific. It should be mentioned that the GDP effect on incorporation into protein has been found for six separate labeled amino acids.

INCORPORATION INTO NUCLEOPROTEIN PARTICLES

Electron micrographs of these microsome fractions were made by Dr. Jerome Gross, with the demonstration of two main particulate types — one large and irregular, and the other small, dense, and relatively homogeneous (Keller *et al.*, '54). If the microsome pellet is suspended in 0.5% sodium deoxycholate, the bulk of the protein dissolves, but the small, dense particles may still be collected by centrifugation, in a rather pure state (Littlefield *et al.*, '55). These particles have a diameter in unfixed, unshadowed, specimens of around 240 A, a ribonucleic acid/protein ratio of 1, and may be termed "ribonucleoprotein particles." When microsomes, labeled in a cell-free system, are separated in this way, the preponderance of the radioactivity is found in the ribonucleoprotein part of the microsomes. Similarly, with *in vivo* experiments, 3 minutes after the intravenous injection of C^{14}-leucine or C^{14}-valine, the highest specific activity occurs in the ribonucleoprotein fraction of the microsomes. Thus, when a small

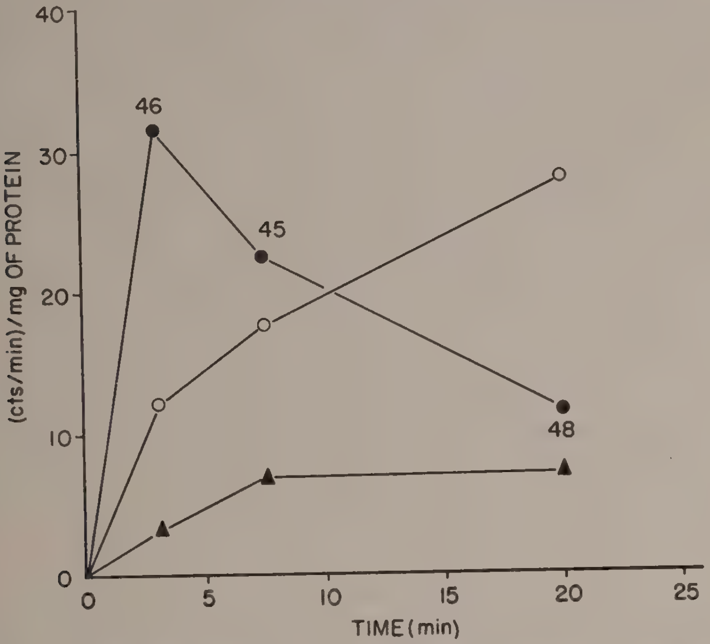

Fig. 1 *In vivo* incorporation of a small dose of leucine-C^{14} into the two components of the microsomes and into the soluble protein of the cell. DL-Leucine-C^{14} [0.25 μmole; 2.3 × 10^7 (cts/min)/mg] in 0.5 ml of isotonic saline was injected intravenously at time zero into a 270-g rat. The percentage of RNA by weight of each deoxycholate-insoluble sample is indicated. The percentage of RNA averaged 2.1 in the deoxycholate-soluble fractions of the microsomes and 1.7 in the soluble fractions of the cell. This percentage of RNA by weight means percentage of RNA in the combined RNA plus protein. Lipid, glycogen, and salts are not included (from Littlefield *et al.*, '55). ○, deoxycholate—soluble; ●, deoxycholate—insoluble; ▲, soluble protein of cell.

amount of radioactive leucine is injected into a whole rat, this ribonucleoprotein fraction becomes very rapidly labeled, then rapidly loses its radioactivity (fig. 1). When a large amount of radioactive leucine (or valine) is injected, a constant high level of intracellular free amino acid radioactivity is maintained. The same small fraction of the ribonucleoprotein becomes labeled, and remains labeled by apparent

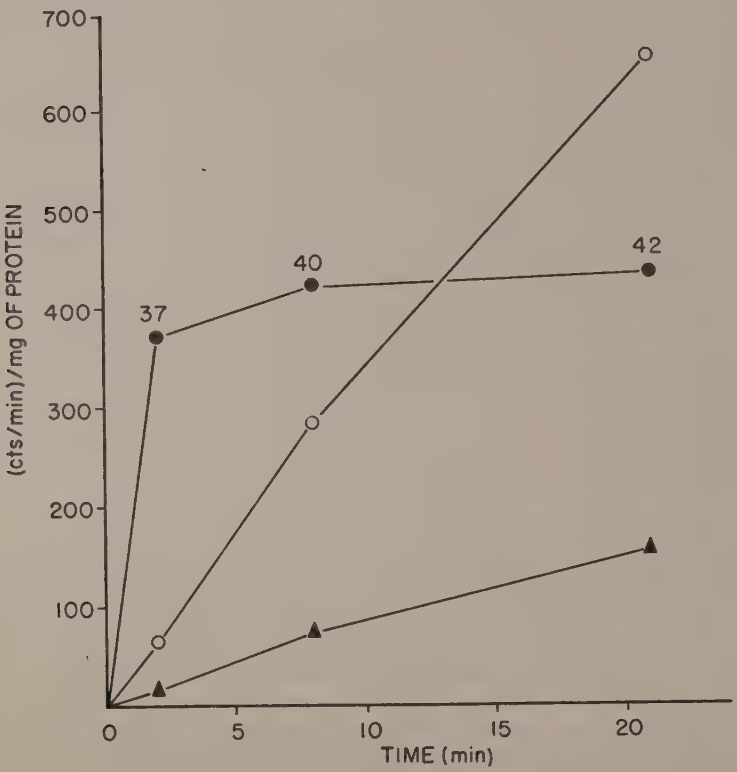

Fig. 2 *In vivo* incorporation of a large dose of leucine-C^{14} into the two components of the microsomes and into the soluble protein of the cell. DL-Leucine-C^{14} [100 μmoles; 2.2×10^6 (cts/min)/mg] in 2.0 ml was injected intravenously at time zero into a 300-g rat. The percentage of RNA by weight of each deoxycholate-insoluble sample is indicated. The percentage of RNA averaged 2.3 in the deoxycholate-soluble fractions of the microsomes and 1.9 in the soluble fractions of the cell (from Littlefield *et al.*, '55). ○, deoxycholate—soluble; ●, deoxycholate—insoluble; ▲, soluble protein of cell.

continual synthesis and loss of this rapidly turning over protein or large peptide fragment (fig. 2). It can be estimated that approximately 1% of the protein of the ribonucleoprotein participates in this rapid labeling process. These findings provide new and more direct evidence for the participation of ribonucleic acid (RNA) in protein synthesis.

Some of the electron micrographs of ribonucleoprotein particles made for these studies suggest a heterogeneity of the particles. It is possible, therefore, that much more than 1% of the protein of certain particles may become labeled, and others may be inert. The problem of further fractionation of the ribonucleoprotein particles is a challenging one, which will require improved fractionation techniques.

NATURE OF THE INCORPORATION REACTION

General comments. In the cell-free system, once C^{14}-leucine or C^{14}-valine has become incorporated into microsome protein, it has not been possible to ''wash it out'' with large quantities of inert leucine or valine. The incorporation is thus a relatively irreversible step on the path to protein synthesis, and differs from the exchange process described by Gale and Folkes ('55) for incorporation of C^{14}-glutamic acid into protein (and possibly glutathione) in the fragmented *Staphylococcus aureus.*

During the last few years the term ''incorporation,'' as applied to amino acids and protein has begun to acquire a more precise meaning than it had earlier (Borsook, '50; Zamecnik, '50). The findings of Peterson and Greenberg ('52) of conversion of C^{14}-glycine to C^{14}-serine and formation of phosphatidyl-C^{14}-serine in homogenates has emphasized the importance of washing out contaminating lipid compounds from trichloroacetic acid-precipitated proteins. It has likewise been found necessary to wash such protein precipitates with mercaptoethanol or a similar disulfide-splitting compound in order to remove glutathione and other sulfur amino acids, a procedure that should be used where C^{14}-

glutamic acid, C^{14}-glycine, or sulfur amino acids are being used (Melchior and Tarver, '47).

The observation of Schweet and Borsook ('53) that C^{14}-lysine is incorporated into guinea pig liver protein in a soluble, cell-free system without added energy donors had also been a puzzle. It is now clear from partial protein degradation studies (Schweet, '55) that the lysine is bound to the protein by its ε-amino group, and is not directly related to protein synthesis.

The use of amino acid analogs had been interpreted (Greenberg, '54) as providing evidence that incorporation of labeled amino acids into protein may be an exchange process, not necessarily a measure of protein synthesis. It has been found, however, that S^{35}-ethionine is actually built into protein (Tarver and Gross, '55) in place of methionine. The antagonist here acts as a competitive substrate, rather than as a competitive inhibitor. Reinterpretation of these results favors *de novo* synthesis rather than exchange (Rabinovitz and Olson, '55).

Criteria used in this study. The best criterion that the labeled amino acid is combined in α-peptide linkage in the labeled protein is the demonstration of radioactive α-peptides on partial hydrolysis of the protein (Steinberg and Anfinsen, '52; Askonas *et al.*, '54).

In this laboratory, the rate of release of C^{14}-leucine from microsome protein labeled in a cell-free system has been compared with the rate of release of the bulk of the leucine from protein (fig. 3). The specific activity of the leucine released by acid hydrolysis does not change, between 15 and 100% hydrolysis (Zamecnik and Keller, '54). This is a strong indication that the labeled leucine is bound in microsome protein in the same type of linkage as is the unlabeled leucine, although it might still be in peptide linkages other than alpha. After incomplete acid hydrolysis, peaks of ninhydrin color and radioactivity appeared at points different from the sites of emergence of known amino acids. One such peptide peak, designated "A" on the curve for 58% hydrolysis, was pooled, concentrated, and subjected to further chromatographic separation by Anne Harris (this laboratory). In order to minimize the possibility of peptide rearrangements during hydrolysis of the protein (Sanger and Thompson, '52), with production of amino acid sequences not present as such in the original protein, hydrolysis at a lower temperature was employed in further studies. To facilitate identification of an individual peptide, a large batch of labeled microsome protein was prepared. The A peak was chromatographed on a Dowex-50 column. From the Dowex-50 eluate,

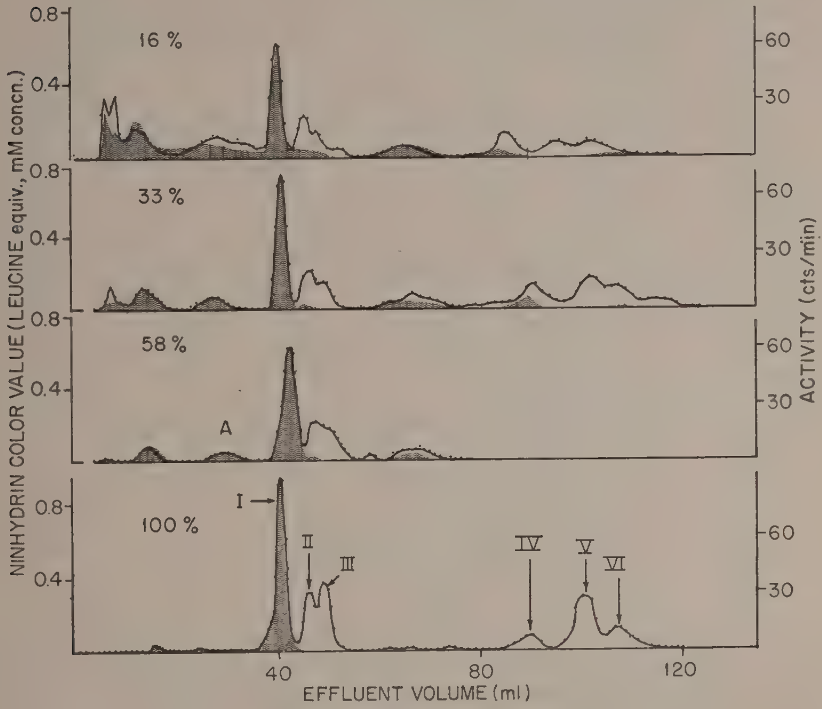

Fig. 3 Aliquots of microsome protein were hydrolyzed in sealed tubes in 2 *N* HCl, and subjected to starch chromatography, as previously described. The A peak was selected for further chromatography (fig. 4). The constancy of specific activity in the leucine peak may be seen.

Peak I, leucine; II, phenylalanine; III, isoleucine; IV, methionine; V, valine; VI, tyrosine.

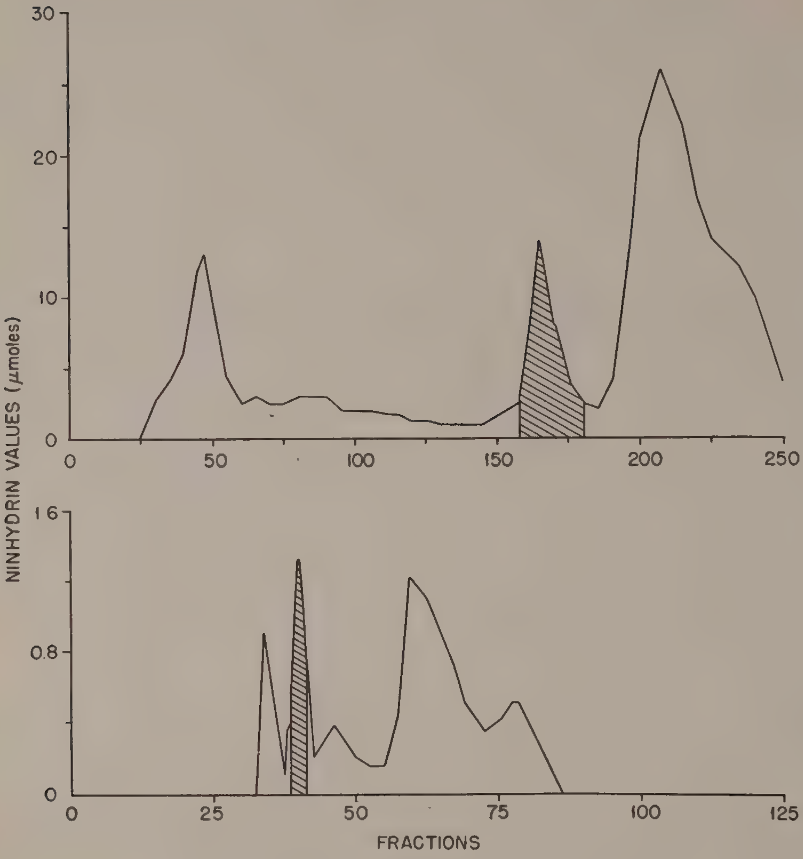

Fig. 4 Further fractionation of the A peak.

Upper graph. Peak A is shaded. Protocol: Washed microsome protein hydrolyzed 12 N HCl; 50° C.; 5½ days. Starch column. Approximately 50% of leucine released.

Lower graph. Shaded peak concentrated and then chromatographed on Whatman No. 4 paper, 2,4,6-collidine saturated with water being used in the vertical direction, and 100 parts of secondary butanol to 40 parts of 3% ammonia in the horizontal direction. Protocol: Dowex-50 column; buffer at pH 4.7.

(1) The shaded peak on lower graph represents one spot on paper.

(2) Only isoleucine and leucine yielded on complete hydrolysis.

(3) Use of DNP, followed by hydrolysis, indicates the sequence: isoleucylleucine.

a sharp peptide peak was selected, and was found by further hydrolysis and paper chromatography to be a peptide or peptides containing isoleucine and valine in addition to leucine-C^{14} (fig. 4). In a similar experiment, the sequence isoleucyl-leucine was identified from this type of peak by means of the DNP technique and further hydrolysis. This acid hydrolytic procedure was chosen so as to destroy all but the most acid-resistant peptides of a complex mixture of peptides. These results are in conformity with well-known observations that isoleucyl and valyl peptides are very resistant to acid hydrolysis, and thus would be expected to survive under such rigorous conditions. This painstaking analysis, plus the background work on selection of appropriate conditions for the peptide separations, has therefore provided an affirmative answer to the question of whether a labeled amino acid is built into protein (or a large peptide) in this cell-free system. It does not, however, provide blanket assurance that the same holds for all cell-free systems.

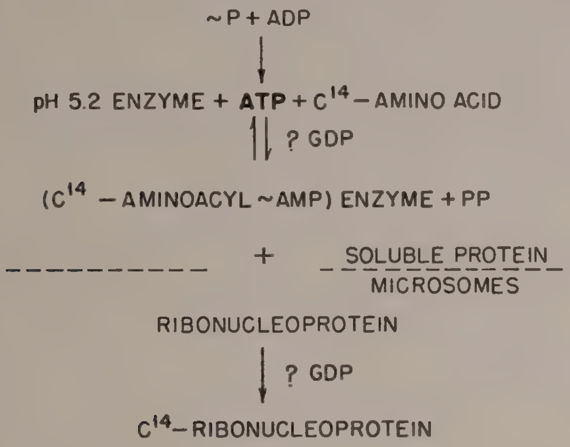

Figure 5

SUMMARY

These results are summarized schematically in figure 5. In the rat liver homogenate-soluble protein fraction, a general amino acid activation mechanism has been found. In the presence of ATP and soluble enzymes, the amino acids are converted to aminoacyl-AMP compounds. These activated amino acids are built into proteins (or large peptides) in ribonucleoprotein particles located in the microsome fraction of the liver cell cytoplasm. GDP, GTP, or a derivative thereof play an essential role, in some unknown way, in this over-all cell-free incorporation process. Once the C^{14}-amino acid is incorporated into protein in the cell-free system, the process appears to be irreversible. From partial acid hydrolysis of labeled microsome protein, a labeled peptide has been isolated and identified, indicating α-peptide bond formation. This finding provides assurance that this sequence of steps is of significance in the synthesis of protein.

ACKNOWLEDGMENT

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GENERAL DISCUSSION

CHARGAFF[3]: Dr. Zamecnik, does your soluble 100,000 x *g* supernatant fraction contain RNA? Also, would we not visualize it as playing a role, together with the microsomal particles, in what you consider to be evidence of protein synthesis?

ZAMECNIK: One cannot say that the RNA present in the 100,000 x *g* supernatant protein fraction does not play a special role in the incorporation process. It can be stated that the concentration of RNA in this fraction is low, around 1.8% of the combined protein-RNA weight. In the whole microsome fraction, the corresponding figure for RNA is about 13%, and the RNA figure is 40–50% in the ribonucleoprotein particles. With high-speed centrifugation, it is hard to sediment 100% of the microsome fraction in a finite time.

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[3] E. Chargaff, Columbia University.

The amino acid-activating step of Hoagland's system, which occurs in this 100,000 x *g* supernatant protein fraction, is not inhibited by ribonuclease.

Your point remains, Dr. Chargaff, that there may be some critical nucleic acids in this 100,000 x *g* supernatant fraction.

CHARGAFF: May I ask one more question in this connection? Do you have any information on the effect of various nucleotide diphosphates, other than GDP, on this incorporation process? Also, is ATP required in the first step?

ZAMECNIK: We did try other diphosphates, without stimulation to the incorporating system.

Another thing we should like to do, of course, would be to employ a GTP-generating system that does not go through ATP. This would enable one to determine for certain whether ATP must react first with the amino acids, as appears to be the case. It is possible that a GTP derivative plays a transfer role at this point, with the aminoacyl-AMP being converted to aminoacyl-GMP or -GDP. If such is the case, a direct GTP-generating system would not be able to replace the ATP-generating system. We have tried a succinyl-coenzyme A mitochondrial preparation for generation of GTP (with the help of Dr. Rao Sanadi), without definitive results.

According to Dr. Hoagland's work, ATP is required in the amino acid-activating step, and cannot be replaced by other triphosphates.

SPIEGELMAN[4]: I should like to make two comments: I should like to make one addition to Borsook's model, which summarizes very beautifully all the facts. Whatever the nature of the template may be, and we assume it is RNA according to the model, I would suggest that it cannot function as a passive mold. The reasons for the amendment stem from a series of experiments, two of which I should like to indicate briefly, performed by Dr. Ruth Ben-Ishai in our laboratory. It is found that complete inhibition of enzyme formation is attained by introducing an inducer and a compound (5-OH-uridine) that can prevent RNA synthesis simultaneously. If the cell is first allowed to start making enzymes and then the hydroxyuridine is added, again inhibition of enzyme synthesis is obtained. Thus, since the cell has already accumulated presumably the right RNA for the formation of the particular enzyme being studied, it is not allowed to continue synthesizing this enzyme when the synthesis of new RNA is prevented.

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[4] S. Spiegelman, University of Illinois.

The same type of experiment can be performed without the use of a specific agent interfering with RNA synthesis with yeast. Yeast contains a large pool of free nucleotides and their derivatives. Such cells may be partially induced, to the stage where about half of the enzyme they can eventually make has been synthesized. If their free nucleotide pools are now depleted and the inducer reintroduced, no further synthesis of enzyme occurs. If, however, the depleted nucleotide pool is replenished, reintroduction of the inducer results in the continuance of normal enzyme formation.

Both these experiments suggest that either protein synthesis is mandatorily coupled to the synthesis of the new RNA molecules, or the destruction of an RNA molecule is mandatorily coupled to its function as a protein-synthesizing machine. In either case, a passive mold is eliminated.

With respect to the beautiful experiments of Dr. Zamecnik, I would suggest that he not be too much worried about the induced enzyme formation data if he can continue performing experiments such as those he described. I might note that, although in our earlier work on subcellular fractions we were unable to obtain enzyme formation in protoplasts, we have since succeeded in demonstrating that this is possible. Indeed, enzyme formation can be obtained which compares very favorably with that of intact cells held under the same conditions. The difficulty is to find a suitable stabilizing medium and proper supplementation. We have found that such things as sucrose prevent the synthesis of the enzyme being followed, and, after some effort, found also that 0.5 M Na_2HPO_4 is a suitable stabilizer. In addition, these protoplasts required an elevated supply of amino acids, inducer, and HDP if adequate enzyme formation was to be observed.

ZAMECNIK: We would like to study induced enzyme formation, too; but that reminds me of a story Dr. Hotchkiss told me of a man who wanted to use a new boomerang but found himself unable to throw his old one away successfully.

ROBERTS[5]: I should like to describe some experiments that Dr. Britten and I have carried out during the last few months on amino acid incorporation by growing *Escherichia coli.* These experiments are similar to those of Dr. Zamecnik and have a bearing on the mechanisms of protein synthesis discussed by Dr. Borsook.

Our basic procedure is to add a very small quantity of highly radioactive amino acid (25% C^{14}) to a culture of *E. coli* growing in a glucose-ammonia medium. Samples are then taken at intervals. In alternate samples, the total incorporated is measured by filtering off the medium and the TCA-insoluble fraction. Chromatograms show that the radioactivity of the TCA-insoluble material is mostly bound in protein, whereas the radioactive material extracted by TCA (trichloroacetic acid) is the free amino acid.

The radioactivity of the TCA-insoluble fraction increases almost linearly with time until the supply of amino acid is exhausted and then remains constant. In a typical experiment, this would require 3–5 minutes. In contrast, the TCA-soluble fraction rises more rapidly at first and then decreases as its material is transferred to the protein.

The amino acids are evidently adsorbed by the cells as the concentration per milliliter of cells reaches several hundred times the concentration per milliliter of medium. Furthermore, several features of the adsorption process indicate that it is an early step in protein synthesis: (1) Presaturation of the adsorption sites with a C^{12}-amino acid causes a delay in the incorporation of a subsequently added C^{14}-amino acid. This indicates that adsorption is a part of the mechanism for protein synthesis rather than a storage mechanism. (2) The adsorption is specific. The addition of sixteen other amino acids at several hundred times as great concentration had no effect on the adsorption of C^{14}-proline and its incorporation into protein. (3) The adsorption process requires energy. There is little adsorption by glucose-starved cells or by cells blocked by DNP. (4) Adsorption, but not incorporation into protein, can occur when protein synthesis is blocked by chloroamphenicol, nitrogen deficiency, or by lack of an amino acid in a deficient mutant.

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[5] R. B. Roberts, Carnegie Institution of Washington.

These observations agree with the theory that amino acids are ordered by adsorption on specific activated sites of a template, possibly RNA, prior to the formation of a peptide chain. However, if the amino acids were activated before adsorption, as suggested by Dr. Borsook, we might expect that there would be a competition for the available energy and that the presence of other amino acids would reduce the adsorption of proline.

Most of these experiments have been carried out with proline and methionine but some of the characteristic features have been checked with alanine, valine, glutamic acid, phenylalanine, tyrosine, arginine, and lysine. (This work was reported at the spring 1955 meeting of the National Academy of Sciences; a more complete report will be published in the Yearbook of the Carnegie Institution of Washington.)

COWIE [6] : Investigations carried out in our laboratory on the kinetics of formation and utilization of the "free amino acid pool" in *Torulopsis utilis* have shown that materials from the medium flow through a pool of metabolic intermediates into constitutive proteins and nucleic acid. The pool of amino acid intermediates is extracted by cold TCA and contains 13% of the total carbon of the cell. Observations of the incorporation of C^{14}-fructose in exponentially growing cells have shown that the rate of formation of the pool exceeded the growth rate by 4.75 times. This rapid incorporation in excess of the growth rate is required if the TCA-soluble pool incorporates carbon in order to keep the pool filled and also to provide for transfer of carbon to the protein and and nucleic acid of the cell.

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[6] D. B. Cowie, Carnegie Institution of Washington.

Five minutes' immersion of exponentially growing cells in a medium containing C^{14}-fructose yields cells having 70% of the radioactivity in the TCA-soluble fraction. When these cells are washed and transferred to a medium containing C^{12}-fructose, the pool of intermediates rapidly loses radiocarbon and the proteins and nucleic acid show a corresponding increase in radioactivity. This transfer of radiocarbon from the pool to protein and nucleic acid occurs at the same rate even when exogenous amino acids at high concentrations are present in the C^{12}-fructose medium. Thus the preformed, labeled amino acids of the TCA-soluble fraction are utilized in preference to the exogenous, nonradioactive supplements. On the other hand, when *T. utilis* is grown in a medium containing both C^{14}-fructose and C^{12} amino acids, the exogenous amino acids reduce the incorporation of radiocarbon into the pool as well as into the protein and nucleic acids of the cell.

These observations demonstrate that exogenous amino acids can compete with fructose in the biosynthesis of cellular constituents. However, the endogenous amino acids already present in the pool acting as precursor materials for protein and nucleic acid synthesis are not replaced by exogenous amino acids. It is concluded that the amino acids of this pool are not "free amino acids" available for exchange or loss by diffusion, but rather are bound in an organized pool. Cold TCA disrupts the organization and liberates free amino acids. The nature of the association between amino acid intermediates and nondiffusible cell components has not been established.

VINCENT[7]: Both Dr. Borsook and Dr. Zamecnik have suggested that there should be two RNA fractions in the cell, with differing characteristics. I have in progress some studies on the nucleolus of the starfish oocyte that strongly suggest that this organelle contains two classes of RNA differing widely in both solubility and metabolic activity. The evidence is as follows:

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[7] W. S. Vincent, State University of New York, Syracuse.

1. Microspectrophotometric comparisons were made of the nucleotide content of individual *in situ* and isolated nucleoli. The *in situ* nucleolus of a given size always contains more RNA than the isolated nucleolus of the same size. During the early stages of the growth of the oocyte, this difference is proportional to total nucleotide content; at the time when the oocyte enters its major period of protein synthetic activity, the difference becomes a constant value, even though the total content of RNA in the nucleolus continues to increase some 4- or 5-fold. This difference does not exceed 10%.

2. P^{32} incorporation into the nucleolar RNA was measured by both autoradiographs and direct analyses of isolated nucleoli. In the autoradiographs, the specific activity of nucleolar RNA is many times that of the cytoplasmic RNA; in isolated nucleoli, specific activities of the RNA fractions of nucleolus and cytoplasm are equal. These results indicate that a large proportion of RNA-P^{32} is lost during the isolation procedure.

3. Autoradiographs of distilled water homogenates of starfish oocytes made at intervals during isolation procedures have shown that nucleoli contain progressively less radioactivity as isolation proceeds.

These data suggest that the nucleolus contains two classes of RNA, one a soluble, metabolically very active, fraction, representing only a small portion of the total nucleolar RNA. The other is a less active, tightly bounded fraction, apparently incorporated into the protein structure of the nucleolus. One exciting implication of the active, or labile, form would be that it is involved in the transfer of nuclear "information" to the synthetic centers of the cytoplasm.

Simpson [8]: I should like to describe some experiments, performed in collaboration with Dr. John McLean, that bear closely on the work described by Dr. Zamecnik. We have been studying the *in vivo* incorporation of labeled amino acids into

[8] M. V. Simpson, Yale University.

the proteins of the cytoplasmic structures of rat liver and rat skeletal muscle. Animals were sacrificed at intervals of 2 minutes to 3 hours after intravenous administration of leucine 1-C^{14} or phenylalanine 3-C^{14}, and the radioactivity determined for the proteins of the various cell fractions. We have found, in accord with reports from other laboratories, that, in liver, the protein of the microsome fraction has a considerably higher rate of uptake of labeled amino acid than that of any other tissue fraction; the uptake by the microsomal protein was found to be 4–8 times as high as that by the mitochondrial (lowest) fraction. In muscle, however, even when measured at extremely short time intervals, no substantial difference could be demonstrated between the incorporation into the proteins of the mitochondrial and the microsomal fractions, the two highest fractions. The rate of incorporation into these fractions is about twice that into the myofibril or the soluble fractions. These experiments suggest that in muscle, the mitochondrion itself or some other component of the mitochondrial-rich fraction, plays a highly important role in protein synthesis.

HALVORSON [9] : I would like to comment on several features of induced enzyme synthesis in yeast.

Although RNA levels are constant during synthesis in nongrowing cells, experiments involving P^{32}- or C^{14}-glycine incorporation into RNA nucleotides indicate that these incorporations necessarily precede enzyme synthesis. Ultraviolet irradiation, which inhibits both these processes, not only inhibits the utilization of the free amino acid pool but also stimulates pool rises. Cells that have been briefly exposed to labeled amino acids, starved, and irradiated possess pools composed largely of labeled amino acids that had previously disappeared from the pool. It would be interesting to see whether the nucleic acid damage and amino acid release are related.

The other comment is concerned with the posttemplate stage of Dr. Borsook's mechanism. We observed in fully induced

[9] H. Halvorson, University of Michigan Medical School.

yeast that a fraction of the α-glucosidase was bound to a particulate fraction. It could be solubilized by incubating the washed particles. The amount of bound enzyme is related to the stage of enzyme synthesis, being absent in the early stages of induction. Conditions that inhibit protein or nucleic acid synthesis or treatment of the particles with RNAse or DNAse do not effect the release. This bound enzyme may represent either a late stage in the enzyme-forming system or an enzyme reservoir.

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NUCLEOPROTEINS

ROUND-TABLE DISCUSSION

A. L. Dounce, *University of Rochester*
G. Gamow, *George Washington University*
S. Spiegelman, *University of Illinois*
P. Newmark, *University of Kansas*
D. Harker, *Polytechnic Institute of Brooklyn*
M. Soodak, *Massachusetts General Hospital*

Dounce: I should like to say a word about template theory that may lead rather easily to the discussion of nucleoproteins. My interest in templates, and conviction of their necessity, originated from a question asked me on my Ph.D. oral examination by Professor J. B. Sumner. He inquired how I thought proteins might be synthesized. I gave what seemed the obvious answer, namely, that enzymes must be responsible. Professor Sumner then asked me the chemical nature of enzymes, and when I answered that enzymes were proteins or contained proteins as essential components, he asked whether these enzyme proteins were synthesized by other enzymes and so on *ad finitum.*

The dilemma remained in my mind, causing me to look for possible solutions that would be acceptable, at least from the standpoint of logic. The dilemma, of course, involves the specificity of the protein molecule, which doubtless depends to a considerable degree on the sequences of amino acids in the peptide chain of the protein. The problem is to find a reasonably simple mechanism that could account for specific sequences without demanding the presence of an ever-increasing number of new specific enzymes for the synthesis of each new protein molecule.

A possible solution, which has been advocated for some time, is to assume the presence of templates or patterns in the cell, each of which should correspond to at least one peptide chain, and to determine the kind and quantity of each amino acid present in this chain, as well as the order of arrangement of these amino acids. However, it must not be forgotten that nothing has really been accomplished by such a postulate unless the template is of such a nature that its action can be understood on a molecular basis, and in particular, how it can be reproduced. We should avoid the construction of a hypothetical template so complicated that its duplication would be considerably more difficult than its alleged action as a template.

The simplest possible template model would itself be a peptide chain, but such a template is rather unsatisfactory since there seems to be no convincing way to make the template align the incoming amino acids in the proper order. There are no regularly recurring functional groups in a peptide chain, other than the peptide bonds themselves, that might complex and hold the incoming amino acids prior to their being united to form a new peptide chain. Moreover, peptide bonds are rather inert and usually do not enter into reactions with organic molecules unless they are broken in the process. Nucleic acid, on the other hand, seems to offer just the sort of template that is demanded, with the remaining –OH on each doubly esterified phosphate group acting as the required recurring functional group. Moreover, the duplication of the nucleic acid molecule would seem less difficult to explain than its possible action as a template.

If a nucleic acid molecule, for example, one of ribonucleic acid (RNA), is to act as a template, there must be enough information stored in the polynucleotide chain, composed of only four different mononucleotides, to determine the order of arrangement and relative amounts of some twenty different amino acids in a peptide chain. Enough information could thus be stored only if a combination of nucleotides could determine the position of a given amino acid. Two possible classes of mechanisms might be envisioned for the transfer of information from a polynucleotide chain to a peptide chain.

The first of these is that only the combinations of nucleotides would determine the positions of the amino acids. In my hypothetical mechanism for peptide chain synthesis, which was so charitably treated by Dr. Borsook, three neighboring nucleotides were assumed to determine the position of a given amino acid, and it was assumed that each individual nucleotide phosphate carried an amino acid. In such a system, the triad of nucleotides determining a given amino acid overlaps by one with the triad of nucleotides determining the next amino acid of the sequence. It can easily be shown that this overlapping would limit to four the number of possible neighboring amino acids that could occur on one side of a given amino acid, if it is assumed that only one triad can correspond to a given amino acid. However, the results obtained for the sequences of amino acids in the peptide chains of the insulin molecule show that a given amino acid can have more than four possible neighbors on a given side, even in such a relatively simple protein.

Dr. M. Morrison of the University of Rochester, who first called this difficulty to my attention, has indicated that there is a way out, which is to assume that the location of a given amino acid can be determined by more than one nucleotide triad. If direction in the polynucleotide chain is considered important, there are 64 possible nucleotide triads and only about 20 amino acids to be accounted for; it could therefore be assumed that an amino acid is directed by any one of three nucleotide triads. (There would still be four triads to spare with this scheme.) There would be four possible neighboring amino acids on one side of a given amino acid for each of the three nucleotide triads determining this amino acid, making a total of twelve possible neighbors on the given side. Whether or not even twelve possible neighbors on a given side are sufficient remains to be seen. This might depend on whether the system of assignments were the same throughout nature, or varied from species to species.

The second class of possible mechanisms by which a nucleic acid molecule might determine the order of occurrence of amino acids in peptide chains is one in which both a combination of nucleotides and the preceding amino acid determine the incoming amino acid. (This type of mechanism was thought of during discussions of the problem by Dr. Morrison, Mr. K. J. Monty, and me at Rochester, and I am informed by Dr. Gamow that it has also been suggested by the physicist, Dr. Edward Teller.) Such a mechanism would require two nucleotides and one amino acid for determining the identity of a given incoming acid. It leads to a very large number of possible assignments in terms of the triads composed of two nucleotides and one amino acid, thus obviating the problem of neighbors. The amino acids would have to go onto the template one by one, however, and this might present difficulties from the standpoint of kinetics. If there were some flexibility that would allow the amino acid at the beginning of the sequence to be any one of possibly four, then the template should be capable of synthesizing four different peptide chains. This statement can easily be verified by the procedure of trial and error.

GAMOW: Dr. Dounce assumes that the *order* of bases within each triplet is essential for determining which amino acid will be attached at that section of template, and obtains in this way *64* possibilities. If, however, it is assumed that *the order is of no importance,* there are only *20* possibilities, which helps to explain in a natural way the observed number of amino acids. (Those above 20, e.g., hydroxyproline, are probably formed after incorporation into the protein.)

I should like to mention briefly a possible way of assigning individual amino acids to different base triplets. In tobacco mosaic virus (TMV), percentages of bases in RNA and of amino acids in the corresponding protein have been measured. By the observed abundances of bases, the expected abundances of various triplets, such as (Ad, Ad, Ur) or (Cy, Gu, Ur), can be calculated, and plotted in decreasing order. In a comparison of that plot with the corresponding plot for the observed abundances of amino acids, it is found that both curves run almost parallel, thus leading to a unique assignment between amino acids and base triplets. Unfortunately, a similar procedure carried out for turnip yellow mosaic virus (TYMV) leads to assignments different from those given by TMV. I believe, however, that it may be possible to find some "intermediate" set of assignments that would satisfy, with sufficient approximation, both TMV and TYMV data.

Dounce: One difficulty with decoding is that too few peptide sequences are known at the present time. Another possible difficulty is the question raised as to whether the coding must be the same throughout nature. I am now more inclined to agree with Dr. Gamow, however, that it should be the same. There is also a question as to whether enzymes are needed to attach the amino acids to the nucleic acid template. Considerable thought should be devoted to this point. In general, a system of assigning amino acids to nucleotide triplets would seem to present many difficulties in regard to the "neighbor" problem, if the molecular geometry is not ignored. The molecular geometry is most certainly one of the most important features of the whole problem.

In spite of the difficulties with nucleotide triplets already encountered, it would not seem wise to be easily discouraged in attempts to set up a workable scheme for the transfer of information from an RNA molecule to a peptide chain, since it now seems rather evident that some kind of template must be involved in a peptide chain synthesis, and RNA still seems to be the best candidate for the template molecules.

Spiegelman: I brought up the question of interpretation of this data because I know, of course, that it is based on as much information as can possibly be gathered from the literature. However, even if attention is confined to the two known proteins taken from the same species, i.e., obtained from the same animal, this analysis still yields nothing that strongly suggests nonrandom order. If I were asked to wager on what would happen with subsequent adequate information, I would say that your conclusion is probably correct but the other possibility still remains.

Gamow: I am not sure that Poisson analysis is sensitive enough to rule out a single overlap in base sequence. I have constructed random sequences of bases by using a well-shuffled pack of canasta playing cards, and calling hearts Ad, spades Gu, etc. It looks like: Ad, Ad, Cy, Ur, Ad, Gu, Cy, etc. If one uses nonoverlapping triplets, e.g., (Ad, Ad, Cy), (Ur, Ad, Gu), one gets an ''artificial protein'' that satisfies Poisson distribution. Use of a *double overlap,* e.g., (Ad, Ad, Cy), (Ad, Cy, Ur), (Cy, Ur, Ad), gives a sequence of amino acids that definitely deviates from Poisson rule. However, use of a *single overlap,* e.g., (Ad, Ad, Cy), (Cy, Ur, Ad), (Ad, Gu, Cy), gives a sequence that, being subjected to Poisson analysis, does not show any apparent deviation from randomness.

Newmark: Consideration of the structures of the two plant viruses discussed by Dr. Gamow may help in applying template hypotheses.

TMV is a long rod, whereas TYMV is globular in shape. The protein and nucleic acid portions of TMV may be split apart with dilute alkali. The protein obtained is homogeneous by several criteria. It has a molecular weight under 50,000, and can be readily repolymerized into noninfectious, rod-like particles that closely resemble the virus but contain no nucleic acid. The chemical polypeptide subunit obtained by degradation of TMV with detergent appears to have a molecular weight of 17,000, and is composed of about 150 amino acid groups. By comparison, the nucleic acid portion of TMV has a ''molecular weight'' of 2–3 million, which — no repeat units being assumed — represents about 10^4 nucleotides. If this view is correct, then the information in 10^4 nucleotides is presumably necessary for the synthesis of a polypeptide chain of some 150 amino acids.

Existence of a comparable picture has not been shown for TYMV. It has not yet been possible to dissociate the nucleic acid and protein portions of this virus in a manner that permits reaggregation of the nucleic acid-free protein to form particles resembling the intact virus. Furthermore, a specific nucleic acid-free abnormal protein, closely resembling the virus protein and which accompanies virus infection in each case, appears only as a particle resembling the intact globular virus in TYMV; whereas, in TMV, the abnormal protein is isolated as the subunit. It is possible, then, that the TYMV protein is composed of one or, at the most, only a few high-molecular-weight polypeptide chains.

The total amount of nucleic acid per virus particle is about the same for both viruses, i.e., about 10^4 nucleotides. In TMV this represents 6% of the particle weight, whereas in TYMV it represents about 35%.

Since the two viruses appear to be so different in structure, it should not be too surprising that a distribution pattern of the relative quantities of amino acids that holds for TMV does not hold for TYMV.

HARKER: The structure of TMV proposed by Rosalind Franklin does not agree with these ideas on information transfer. The nucleic acid is in the form of a rod about which are wound two strands of protein, presumably α-helices. This structure provides contact with the nucleic acid for only about 40% of the amino acid residues in the protein, and, consequently, only 40% of the sequence in the polypeptide chains could be determined by the nucleic acid. The remainder of the sequence, if determined at all, could be determined only by the intervening amino acid residues.

SOODAK: The vitamins have fallen into a rather consistent pattern. Many members of the group have been found to exist and function in some coenzyme form. It is generally assumed that all the B vitamins will be demonstrated to function in this manner.

The role played by adenine derivatives in catalytic processes was well documented by 1950. Adenylic acid, adenosine diphosphate, adenosine triphosphate, coenzyme I, coenzyme II, flavinadenine dinucleotide, and coenzyme A are all coenzymes containing adenine. The last four mentioned also contain one of the B vitamins. When Leloir and his group (Caputto *et al.*, '50; Leloir, '51) demonstrated the role of uridine diphosphate glucose (UDPG), as uracil derivative, as the coenzyme for the galactowaldenase system, it seemed logical at that time to suggest, as an extension of the pattern set by B vitamins, the possibility that the other purine and pyrimidine bases found in some nucleic acids (e.g., guanine, thymine, cytosine, 5-methylcytosine, and later the 5-hydroxymethyl cytosine of the T-even coliphages) might also prove to be parts of coenzymes.

Extensive evidence for this possibility has already accumulated. Ten uridine diphosphate esters of the same type as Leloir's uridine diphosphate glucose have been isolated. Guanosine diphosphate mannose and three cytidine diphosphate esters have also been demonstrated. The mono-, di-, and triphosphates of adenine, uracil, guanine, and cytosine have been found to occur normally in liver and yeast. That most of these compounds possess special coenzymic functions is becoming increasingly evident. The pattern set by the B vitamins is indeed applicable to the nucleic acid bases.

Four other considerations would seem to strengthen the hypothesis that the B vitamins and the nucleic acid bases play similar roles in metabolism.

1. There are basic similarities in structure between these two groups of compounds. Almost all the B vitamins, like the nucleic acid bases, are heterocyclic compounds containing nitrogen. It is also of interest that nicotinamide in coenzyme I and coenzyme II, the isoalloxazine base in flavin mononucleotide and flavin dinucleotide, and the 5,6-dimethyl benzimidazole of vitamin B_{12} exist in the "nucleotide" form. These bases are linked to their corresponding C_5 residues via a nitrogen atom.

2. The concentration of the B vitamins and the nucleic acid bases in rat liver are similar, of millimicromolar and low-micromolar magnitudes.

3. Many microorganisms have been shown to require nucleic acid derivatives as growth factors or for elaboration of certain products.

4. Warkany ('54) has pointed out the interesting similarities between the congenital malformations induced by maternal vitamin deficiencies and those malformations of genetic origin.

Green's "trace substance-enzyme thesis" ('41) boils down to the view that enzyme catalysis is the only rational explanation of how a trace of some substance can produce profound biological effects. In the light of the five lines of evidence stated and in the light of the "trace substance-enzyme thesis," the hypothesis is put forward stating that all the derivative bases of the nucleic acids are components of coenzymes of the usual size, and that the nucleic acids themselves, or polynucleotides derived from them may also be coenzymes. It is further postulated that one of the major roles of both the ribonucleic and deoxyribonucleic acids is to serve as coenzymes or as source material for coenzyme formation. This precursor material may be as polynucleotides, nucleotides, nucleosides, the bases, ribose phosphates, or even as inorganic phosphate. An ordered release of coenzymes or precursors for coenzyme synthesis at certain times in the life cycle of the cell would determine the rate, the concentration, and the time of formation of coenzymes. These factors would in turn control the rate, the concentration, and time of appearance of cellular substances, be they other coenzymes, proteins, lipids, or carbohydrates. It is postulated that in this manner the nucleic acids act as important controlling influences in the numerous biosynthetic processes occurring in the cell.

Most of the theories concerning the action of nucleic acids involve a nucleic acid template mechanism for protein synthesis. That nucleic acids may also push protein synthesis to completion, or act as a source of energy, or provide material for coenzyme synthesis, has also been suggested. The theory proposed here is essentially not a new idea, but represents a change of emphasis based on a biochemical approach. The suggestion that the nucleic acids may act mainly as coenzymes or coenzyme precursors opens up a broader horizon for investigation.

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STRUCTURAL FACTORS IN PROTEIN DENATURATION

WALTER KAUZMANN
Frick Chemical Laboratory, Princeton University, Princeton, New Jersey

According to current views of protein structure, the synthesis of a native globular protein from its amino acids can be imagined to involve three stages (Linderstrøm-Lang, '52):

Stage 1. The amino acids are joined together by peptide bonds to form long polypeptide chains. Several of these chains may also be linked together laterally by chemical bonds between the side chains, such as cystine disulfide links. Chemical cross links may also be formed between different parts of a single chain.

Stage 2. The polypeptide chain is folded in some regular way. The folds are maintained by the stable and very numerous hydrogen bonds formed between the hydrogen atoms on the peptide-NH group and the oxygen atom of the peptide-CO group. Most of the current discussion of folding is in terms of the Pauling-Corey helices (Pauling *et al.,* '51), there being some strong indirect evidence for these helices in certain proteins. Therefore, it might be appropriate to give this stage of protein synthesis the name "helix formation." It is, however, possible that hydrogen-bonded structural elements other than helices might be involved in some or even in all proteins. Therefore, although frequent mention will be made of helices in this paper, the possible existence of other types of structures stabilized by hydrogen bonds between peptide links should not be completely disregarded.

Stage 3. The helix is then bent into short segments which are packed together in a definite way to form the native globular protein molecule. The structure introduced at this stage may be stabilized by many different kinds of bonds. ''Hydrophobic bonds'' (Kauzmann, '54) are probably very important. These bonds are the result of the tendency of nonpolar side chains such as the benzyl group of phenylalanine, the butyls of the leucines, the propyl of valine, and the indole of tryptophan to adhere to one another because of their low affinity for water. Salt linkages between the positively charged side chains of the basic amino acids lysine and arginine and the negatively charged side chains of the acidic glutamic and aspartic acid residues may also play some role here, though Jacobsen and Linderstrøm-Lang ('49) have shown that they cannot be very numerous in most proteins. Hydrogen bonds may also be involved, but it must be kept in mind that intramolecular hydrogen bonds have to be stable to attack by water if they are to be effective in maintaining the structure when the protein is dissolved. For this reason it does not seem wise to be over-sanguine in invoking hydrogen bonds of all types in explaining protein properties; the unusually stable and abundant hydrogen bonds that form between peptide bonds are probably by far the most important in proteins.

Linderstrøm-Lang ('52) has called the structural element introduced in stage 1 the ''primary structure,'' that introduced in stage 2 the ''secondary structure,'' and that introduced in stage 3 the ''tertiary structure.'' Thus the primary structure is that which is maintained by ordinary chemical bonds, the secondary structure is maintained by hydrogen bonds between peptide linkages, and the tertiary structure is maintained by various other nonchemical bonds. It should be emphasized that this way of looking at protein structure need not imply that proteins are synthesized in precisely the temporal succession of steps that has just been outlined. In fact, it is possible that, for instance, some of the chemical links in the primary structure are formed after the tertiary structure has been completed. The order of completion of the stages in time is not important; the aim is merely to emphasize that, according to contemporary ideas of protein structure, these three levels of structural organization should exist in the native protein molecule.

This paper is concerned with the consequences of the conception of levels of organization on the interpretation of protein denaturation reactions. It also deals with the question of what determines a protein's resistance to denaturation in the light of this concept, and with the question of why different proteins differ in stability. An excellent discussion of protein denaturation by Lumry and Eyring ('54) also starts from this concept of primary, secondary, and tertiary structures, but the concern here is with a somewhat different aspect of the denaturation question than that considered by them.

STRUCTURAL FACTORS AND PROTEIN STABILITY

Any process that alters or destroys the secondary or tertiary structures characteristic of a protein can be called a denaturation process. The ease with which each of these structures is broken down probably depends on many factors. It is known only that it varies a great deal from one protein to another. The following question is pertinent: How much of a protein's resistance to denaturation is caused by the primary structure, especially by the nonpeptide chemical bonds in the primary structure, and how much of this resistance is contributed by the secondary and tertiary structures?

At first, this question seems to be rather basic, but upon further thought, one realizes that its meaning is not entirely clear. Before going into this, however, it is well to recall that the apparent refusal of a chemical reaction to proceed may be for two entirely different reasons: (1) because it is thermodynamically impossible, or (2) because it is too slow to be observed. A distinction may therefore be made between "thermodynamic stability" and "kinetic stability." This brings up another basic question concerning the resistance of a protein to denaturation: Is this resistance caused by thermodynamic factors or by kinetic factors?

The meaning of these two questions can be made clearer by considering an experimental program by means of which they might be answered. If specific reagents could be found for each type of bond involved in the three structures — that is, a reagent that ruptures only the hydrogen bonds responsible for the secondary structure, another that attacks —S—S— links in the primary structure, another that opens up the hydrophobic bonds of the tertiary structure, and so on — and if each of these reagents were applied to a series of proteins, it should be possible to tell which kinds of structures are chiefly responsible for the stability of each of the native proteins. Whether the stability is thermodynamic or kinetic should appear from a study of the rate of denaturation resulting from the rupture of the different kinds of bonds, and also from a study of the reversibility.

Unfortunately, this approach is not easy in practice. The chief difficulty is in finding suitable reagents. A survey of a list of known denaturing agents shows that in most instances it is uncertain which type of structure is attacked by a given agent. In fact, some denaturing agents may act by exerting a stress on all the bonds at once. This may be true, for instance, of denaturation by acids and bases; it has been shown (Kauzmann, '54) that the enormous electrostatic repulsions between different parts of a protein molecule, when a protein is at a *p*H far from its isoelectric point, could easily be sufficient to disrupt both the secondary and tertiary structures without any more intimate attack on the bonds that maintain these structures. [This explanation of denaturation by acids and bases is in direct contradiction to several recent treatments of the *p*H dependence of denaturation rates (Levy and Benaglia, '50; Levy and Warner, '54; Gibbs, '54). In these other theories, it is assumed that special bonds in proteins are required for stability and that these bonds are broken when a hydrogen ion is added or removed. The identity of the presumed bonds is not at all clear. Ordinary chemical bonds that can conceivably be present in a protein do not respond to *p*H changes in the required ranges of *p*H. The atoms involved in the hydrogen bonds between the NH and CO groups of the peptide links are not sufficiently acidic or basic. Other kinds of hydrogen bonds are too weak or are not present in large enough numbers to contribute significantly to the stability of proteins.]

Reagents for primary bonds

The aim might be to discover some reagents that would attack only primary nonpeptide chemical bonds suspected of helping to maintain the secondary and tertiary structures. For instance, it might be supected that cystine disulfide links are effective in helping to keep a protein in its native configuration. This could be tested by exposing the native protein to a reagent known to split disulfide links, such as thioglycolic acid. Any notable change produced in the stability of the protein by this reagent would indicate that —S—S— bonds help in some way to maintain the native structure. Unfortunately, this approach is severely limited by the well-known fact that many chemical bonds in native proteins do not show their normal chemical reactivities. Therefore, it is not yet possible to deduce very much in this way about the role that the nonpeptide chemical bonds in the primary structure play in determining the stability of the native proteins.

Reagents for tertiary bonds

The situation is almost equally uncertain with regard to reagents attacking only the tertiary structure. It is quite conceivable that the secondary structure could survive the rupture of the tertiary bonds as suggested by Lumry and Eyring ('54), giving a solution containing only segments of helices not joined to one another except through the chemical links of the primary structure. As yet, however, there is no evidence that this sort of thing actually takes place, and the conditions under which it would be expected to take place are uncertain. Possibly detergents and organic solvents act on proteins in this way, since they would be expected to weaken the hydrophobic bonds that are probably important stabilizers of the tertiary structure. This matter obviously deserves further experimental study.

Reagents for secondary bonds

Reagents do exist that probably act almost exclusively on the hydrogen bonds that stabilize the secondary structure. These reagents are urea, guanidinium salts, formamide, and similar substances. They are able to form hydrogen bonds with peptide links that have about the same stability as the hydrogen bonds formed between the peptide links themselves. (Lithium salts may also act in the same way, the hydrogen atom on the water of hydration of the lithium ion being presumably a more effective former of hydrogen bonds than the hydrogen atom in ordinary water.) If this is the case, there is in urea and the urea-like substances just mentioned a group of reagents that attack one type of bond specifically. Therefore, by careful investigation of the behavior of proteins in solutions of urea-like substances, some insight might be gained into the role of the secondary structure in maintaining stability.

Unfortunately, the situation is not quite so simple as this. In interpreting the response of a protein to a reagent that breaks the bonds responsible for the secondary structure the following factors must be kept in mind:

The inherent stability of the secondary structures (helices). This factor is now being studied in detail by Schellman ('55a, b) with most interesting results. It would be expected to be nearly constant, the chief variation coming from the length of the helical segments that exist in the native molecule. One long helix should be more stable than several shorter segments of a helix because fewer unsatisfied hydrogen bonds would be present. The amount of proline in the polypeptide chain, and its distribution along the chain is also a factor since proline residues do not fit into the Pauling-Corey helix.

The influence of the tertiary structure. If the current theories of protein structure are correct, then it is hardly conceivable that the tertiary structure could survive the destruction of the secondary structure. Therefore, the resistance of a protein to attack by urea-like substances must be influenced in some degree by the stability of the tertiary structure.

The influence of the primary structure. If a considerable number of —S—S— bonds are present in a protein molecule, and if they have been formed before the helices develop, they could prevent the development of long helical segments. The resulting secondary structure would be thermodynamically less stable than a structure containing well developed helices. On the other hand, the —S—S— cross linkages might be formed after the secondary structure has developed. If there are not too many of them, the thermodynamic stability of the helix would then be only slightly increased; it might also be somewhat more difficult to start the unfolding of the helices, so that the kinetic stability would be increased.

SURVEY OF THE OBSERVED BEHAVIOR OF PROTEINS IN SOLUTIONS OF UREA AND GUANIDINE SALTS

The thermodynamic and kinetic driving forces tending to disrupt the secondary structure can be changed by varying the concentration of urea-like compounds in solution. This makes it possible to classify proteins according to four general types of behavior:

Type I. The thermodynamic and kinetic stability of the native protein is low, although the native protein is stable in water. Such a protein will show signs of some unfolding even at low urea concentrations and it will refold to give more or less its original structure when the urea is removed or diluted. At all urea concentrations the unfolding will be very rapid.

Type II. The thermodynamic stability of native protein is low, but the kinetic stability is high. The protein will therefore unfold at all urea concentrations, but the unfolding is slow except perhaps at high urea concentrations. The protein will not return to a state resembling its original state on removing or diluting the urea.

Type III. The thermodynamic stability is high but the kinetic stability is low. The protein will begin to unfold only at high urea concentrations, but whatever unfolding takes place occurs rapidly at all urea concentrations. The molecule refolds to give more or less its original structure on removing or diluting the urea.

Type IV. The thermodynamic and kinetic stabilities are both high. The protein will unfold only at high urea concentrations, and then only slowly. It returns to more or less its original state on removing or diluting the urea.

This classification is, of course, only relative, and proteins may be found that are intermediate between two or more types. Furthermore, as will be mentioned, there are proteins, one portion of which seems to be of one type and another portion of another type.

Examples of each of these types of behavior are now known from studies of protein denaturation, chiefly by Jirgensons ('52a, b) and in this laboratory. The work on many of these proteins is only preliminary in nature and more detailed studies are planned. Nevertheless, on the basis of what is now known approximately a dozen proteins can be discussed in terms of the preceding classification. Further work may change the assignments that will be made in this paper, especially insofar as they depend on the reversibility, which is the sole means of distinguishing between types II and III. [It has been assumed here that the return of the optical rotation to its original value on removal of the denaturing agent is an indication of the return of the secondary structure to more or less the same state in which it exists in the native protein. Since the viscosity is less directly dependent on the secondary structure and since it may be influenced by aggregation reactions while the protein is unfolded (Frensdorff *et al.*, '53), the viscosity is not used as a criterion of reversibility except where special care has been taken to avoid aggregation.]

Type I protein. Serum albumin is the most definite example of a protein whose secondary structure has little stability of either the thermodynamic or kinetic kinds. When dissolved in urea at concentrations above the relatively low values of 2 or 3 *M*, there is an instantaneous increase in both the optical rotation and the viscosity (Kauzmann and Simpson, '53; Frensdorff *et al.*, '53). Although there may be further time-dependent changes in the viscosity, these have been shown to be the result of aggregation reactions involving the exchange reaction of a sulfhydryl on one molecule with a disulfide on another. The optical rotation in urea solutions is, however, independent of the time. On reduction of the urea concentration, the optical rotation returns to its native value provided the protein has not been heated. This behavior of serum albumin is consistent with the large number of –S–S– cross links in the molecule and can be taken as an indication that these links are formed before the secondary structure is produced. Other work on serum albumin also indicates a very labile structure. Thus Tanford ('52) has found that the titration curve of serum albumin in acid solutions is inconsistent with a rigid structure, and Yang and Foster ('54) have shown that the viscosity and optical rotation increase in acid solutions. Karush ('50) had earlier suggested that the great affinity of serum albumin for dyes might result from a looseness in structure to which he gave the name ''configurational adaptability.''

Type II proteins. Ovalbumin is the best available example of a protein whose stability is kinetic in origin. Immediately after addition to solutions containing up to 7 *M* urea, the optical rotation and viscosity of ovalbumin are identical with the values for the native protein (Simpson and Kauzmann, '53). In 4 *M* urea, however, there is a very slow unfolding. The rate of this unfolding is extremely sensitive to the urea concentration, being proportional to the fourteenth power of the urea concentration, so that in 10 *M* urea it is about 400,000 times as fast as in 4 *M* urea. Guanidine hydrochloride acts in about the same way as urea (Schellman *et al.*, '53), but only one-third the concentration is required to obtain a given rate of unfolding. On removal of the urea by dialysis or dilution, the protein does not regain its original properties.

These results seem to indicate that ovalbumin is of type II. Evidently, it is difficult to start the unfolding in this protein, but once it has started, there is nothing in the way of an inherent stability to impede the process. Since the addition of organic solvents such as methanol, ethanol, acetone, and dioxane markedly increases the rate, as does also the addition of detergent, it might be that the tertiary structure is responsible for this kinetic stability of ovalbumin. If the tertiary structure were unusually compact, this might make it difficult for the urea molecules to initiate the attack on the secondary structure.

Jirgenson's studies ('52a, b) of the effects of guanidine hydrochloride on chymotrypsinogen, lysozyme, pepsin, and serum γ-globulin indicate that these proteins are probably also of type II. The case of lysozyme is especially interesting because, on removal of the guanidine hydrochloride, Jirgenson obtained an optical rotation that was lower than that of the original protein. Since destruction of the secondary structure in other proteins generally results in an increase in the optical rotation, this might indicate that the lysozyme recovered after denaturation by guanidine hydrochloride has a better developed secondary structure than the original native protein.

Type III proteins. In this laboratory, J. Rupley has found that the absorption spectrum and the viscosity of cytochrome c in urea are essentially unchanged up to nearly 10 *M* urea. With the more powerful denaturing agent, guanidine hydrochloride, he found that the spectrum and viscosity underwent instantaneous and marked changes between 3 and 5 *M*, indicative of rapid unfolding. These changes in spectrum and viscosity were reversed on diluting the guanidine hydrochloride. Cytochrome c thus seems to belong to type III, with a thermodynamically very stable secondary structure, which can be rapidly unfolded when the conditions are such that it becomes thermodynamically unstable.

Type IV proteins. In a study of the denaturation of hemoglobin by urea, J. Simko (this laboratory) finds spectroscopic and viscosity changes in 6–8 M urea, which, however, take place at rates that are sensitive to the urea concentration. There is no indication that the molecule unfolds appreciably immediately after the addition of urea, so that there is clearly some kinetic stability, and since other workers have found that the unfolding can be reversed, there seems also to be thermodynamic stability in the absence of urea. (Unfortunately, the optical rotation of hemoglobin is difficult to measure because of the color.)

Proteins of intermediate type. In addition to the proteins mentioned, there are some whose behavior seems to be of an intermediate type. β-Lactoglobulin (Christensen, '52; Kauzmann and Simpson, '53) changes its optical rotation instantaneously at relatively low urea concentrations, but at higher urea concentrations a time-dependent change follows the initial instantaneous increase. The rate of this time-dependent change is very sensitive to the urea concentration, increasing rapidly as the urea concentration is increased. The changes in optical rotation are largely reversible. This seems to indicate that part of the β-lactoglobulin molecule has a structure of type I and another part has a structure of type II or type IV. Serum γ-globulin may also be intermediate between type I and types II or IV because part of its change in optical rotation in urea seems to be instantaneous.

GENERAL DISCUSSION

Bull[1]: Monolayers of protein spread at an air-water surface beautifully illustrate the primary, secondary, and tertiary structures of proteins discussed by Dr. Kauzmann.

Native egg albumin, when spread on a surface of 5% aqueous ammonium sulfate, can exist in one of two extreme conditions and intermediate structures can be produced at will. If the spreading concentration is above about 0.3 mg per m^2, the egg albumin molecules exist as compact discs which occupy about 1 m^2 per milligram. Such molecules, if deposited on a glass or chromium slide, have the property of combining with specific antibody. This compact but spread structure would be recognized as the secondary structure. If the spreading concentration is less than about 0.3 mg per m^2, the egg albumin molecules expand to an area of about 2.5 m^2 per mg, and appear to have lost their ability to combine with specific antibody. This expanded state can be called the primary structure. The transition of the native, tertiary structure to the secondary structure is of great interest. In spite of considerable effort, we have been unable to detect any intermediate structures between the native molecule and the secondary structure existing on an air-water surface, nor have we been able to visualize how the three-dimensional native molecule is converted into the two-dimensional spread molecule.

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[1] H. B. Bull, University of Iowa.

We have turned our attention to protein films spread at water-solid interfaces and have reason to believe that structures intermediate between the native and the secondary structures do indeed exist at such interfaces. This work will be reported in the near future.

PUTNAM [2]: I was surprised to hear Dr. Kauzmann suggest that detergents attack proteins at hydrophobic regions and that very little is known about the mode of interaction — the more so since the serum albumin-dodecyl sulfate system has been so widely studied. Ten years ago Hans Neurath and I (Putnam and Neurath, '45; Neurath and Putnam, '45) demonstrated that the very first attack of anionic detergents on proteins is by a stoichiometric and electrostatic combination with positively charged groups on the protein. Although Klotz ('46) showed that the binding of the first 10–15 anions is statistical, I believe that he would now agree that thereafter the binding departs from statistical theory and definite complexes containing about 50 and 100 anions, respectively, form upon interaction of serum albumin and dodecyl sulfate. This was first established in our laboratory by viscosity and electrophoretic measurements, and is now supported by later ultracentrifuge data. Equilibrium dialysis measurements, which favor a concept of statistical binding at low molar ratios of detergents, are in accord with our interpretation at the higher ratios studied. However, at yet higher ratios, detergent anions may be bound by hydrophobic attractions. (For a detailed description of our picture of this interaction phenomenon, see Putnam, '48.)

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[2] F. W. Putnam, University of Florida, Gainesville.

Although my remarks are directed toward a single system, there is a great deal of evidence that electrostatic binding and denaturing ability have a number of things in common. For example, affinity follows the Hofmeister series, in which may also be noted the denaturing effect of thiocyanate at one end of the series (high affinity) compared to the protective effect of chloride and sulfate at the other end (low affinity). Serum albumin has the highest binding affinity of any protein known and is quite susceptible to denaturation of your type I, whereas γ-globulin, which has low binding affinity, is much more stable (for fuller analysis of this point of view, see Putnam, '53).

EDSALL[3]: According to the work of Luck and his associates, the stabilizing effect of fatty acid anions on serum albumin, as measured by protection against heat denaturation, increases progressively with increase in length of the nonpolar side chain from acetate up through caprylate, each added methylene group in the chain increasing the stabilization factor. Is this also true of dodecyl sulfate at low concentrations, before enough has been added to denature the protein?

PUTNAM: Yes, I believe it is true that the dodecyl sulfate is more effective than the lower fatty acids and I think there is an apparent relation to structure.

EDSALL: The stabilizing effect of such fatty acid anions on albumin is in marked contrast to their effect on several other proteins. For instance, serum γ-globulin is not only not stabilized by these fatty acid anions but actually is less stable in their presence than in their absence.

[3] J. T. Edsall, Harvard University.

KAUZMANN: I cannot believe that electrostatic effects are all-important in determining the binding of anions by proteins. When a long hydrocarbon tail is attached to the sulfate ion to make the dodecyl sulfate ion, the binding is enormously increased. Therefore, the hydrocarbon tail must play an important part in the process. In fact, long-chain molecules not carrying an ionic charge (e.g., decanol) are tightly bound by serum albumin, as are numerous uncharged steroids (Schellman *et al.*, '54).

HAUROWITZ[4]: How do you explain the strong change in optical rotation when gelatin forms a gel?

KAUZMANN: Robinson and Bott ('51) have found evidence for changes in folding (i.e., secondary structure) in synthetic polypeptides which they relate to the changes that occur in the gelling of gelatin. Furthermore, the inhibition of the gelation of gelatin by urea indicates that hydrogen bonds between peptides may be involved. Therefore, I am inclined to believe that there are changes in secondary structure, and that these are responsible for the changes in optical rotation.

SCHWERT[5]: I think there is little direct evidence that bears on the attractive hypothesis of Eyring and Lumry that reversible denaturation is caused by the breaking of tertiary structure. However, some years ago, Max Eisenberg and I measured the sedimentation velocity of reversibly, thermally denatured chymotrypsinogen in a warm centrifuge rotor and also the light scattering of solutions cooled from the temperature range in which the protein is reversibly denatured to room temperature. In no measurement were any differences found in the properties of native and reversibly denatured protein. These observations led us to the rather tenuous hypothesis that reversible denaturation is simply reversible dehydration.

We have made two further observations in this direction. In the temperature range in which the protein is reversibly denatured, there is no change in the optical rotation of the protein, thereby ruling out, in line with Dr. Kauzmann's presentation, changes in secondary structure. Furthermore, the hypothesis of reversible dehydration seems not to have survived, since we have found the same effects in deuterium oxide as in hydrogen water. The only point we can cling to now is that changes in tertiary structure, if they do occur, must be of a very small magnitude.

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[4] Felix Haurowitz, Indiana University.

[5] G. W. Schwert, Duke University.

Edelhoch[6]: Dr. Kauzmann lumped a very large group of proteins into four groups here, all with respect to urea and guanidine denaturation, which affects the secondary and tertiary structures. There are other ways to modify the secondary and tertiary structures; for instance, by heating to 50° or 60°C. Surface denaturation and even alkali and acid will not affect the primary structure. Will your classification of proteins into these four groups hold for these various other methods of causing denaturation?

Kauzmann: Our reason for sticking to guanidine and urea is that we think we know something about the mechanism of their action on proteins. At least we started out thinking we did. We have since found that some of the other types of bonds have an indirect effect. For instance, the hydrophobic groups have an effect on the rate at which urea breaks down the secondary structure.

As to the acid, alkali, surface, and heat denaturation, some day we are going to understand more about the way they act, but I do not think we now know as much about these processes.

Edelhoch: Do you expect that the same proteins will fit into the same classifications, with respect to these other denaturation agents?

Kauzmann: Serum albumin is unfolded by acid instantaneously and reversibly. Ovalbumin is unfolded by acid at a measurable rate and irreversibly. These two proteins therefore act toward acid in about the same manner as they act toward urea. I believe that their behavior with respect to heat is also in line with their behavior in urea. These factors may indicate that proteins tend to fall into the same categories

[6] H. Edelhoch, University of Kansas.

regardless of the means used to unfold them. I should not be surprised if this were to prove to be the general rule, though I am sure that there will be some exceptions.

EDSALL: Hemoglobin does not fit here. Hemoglobin is very readily denatured by acid and it does not fit in the same category here.

KOFFLER[7]: Most of the discussion so far has been on globular proteins, and I should like to make a few comments regarding the stability of some fibrous proteins, namely, bacterial flagella. These spiral structures, possessed only by motile organisms, are regarded as organs of locomotion; after detachment from the cells they can be isolated, by shaking, in relatively pure form by fractional precipitation and centrifugation. Using the rate at which the viscosity of flagellar suspensions decreases at various temperatures as a measure of thermal stability, Adye and Koffler ('53) have shown that flagella isolated from thermophilic bacteria are much more heat stable than flagella isolated from mesophilic organisms. Since the relative stability of flagella from thermophiles suggested the possibility that they had more effective hydrogen bonding than flagella from mesophiles, it became of interest to determine whether these relatively heat-stable flagella are also more resistant to denaturation by urea. Mallett and I have found that the flagella from four mesophilic species lost their native structure when exposed to concentrations of 4–6 M urea; flagella from four thermophilic species, on the other hand, remained intact in 6 M urea. The behavior of flagella regarding heat and urea seems to be an intrinsic property of the flagella and not the result of contaminating protective or labilizing materials present in the preparations used.

KAUZMANN: What criteria did you use on urea denaturation — what properties did you study?

KOFFLER: Decrease in the viscosity of flagellar suspensions was used. This decrease is accompanied by the disappearance of the characteristic flagellar structure in electron micrographs.

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[7] Henry Koffler, Purdue University.

Ellenbogen [8]: We have made observations that seem to indicate that heat denaturation involves the hydrogen bonds referred to by Dr. Kauzmann as the tertiary structure in the chain. Last year, H. B. Klevens and I studied the binding of perfluorooctanoic acid ($C_7F_{15}COOH$) and bovine serum albumin (BSA), and arrived at the conclusion that many interesting phenomena could be explained only in terms of the hydrogen-bonded structure of the protein. Mr. Klevens also observed that BSA, as well as some heat-labile proteins, may be autoclaved in presence of large amounts of the perfluorooctanoic acid without visible coagulation. P. H. Maurer and I are now in the process of studying this apparent stabilization of BSA to prolonged autoclaving (up to 2 hours) by immunochemical and physicochemical techniques. Preliminary results indicate that the heat coagulation of BSA is a function of the duration of heating, the concentration of perfluorooctanoic acid, and the charge on the protein. Heating gives rise to irreversible changes which are small enough to escape detection by physicochemical techniques, yet are not too extensive to cause irreversible coagulation or gel formation. We interpret these findings tentatively in terms of hydrogen bond formation between the protein and fluorine atoms in such a manner as to stabilize what Dr. Kauzmann calls the "tertiary structure." Such a phenomenon could conceivably preserve the gross shape of the protein, since it involves only the surface. Any disruption of internal hydrogen bonds by heating could alter the internal structure sufficiently to be detectable immunochemically, but not to such an extent as to alter the shape of the molecule appreciably.

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CHEMICAL STUDIES ON ENZYMES AND OTHER PROTEINS

H. FRAENKEL-CONRAT
The Virus Laboratory, University of California, Berkeley

The history of structural protein chemistry dates from about 1945. Knowledge is therefore quite spotty and subject to great expansion and probably some corrections. The brilliant tours-de-force that achieved the complete structural elucidation of insulin and ACTH (Sanger and Tuppy, '51a, b; Sanger and Thompson, '53a, b; Bell, '54) have not yet led to a furthering of the understanding of the mode of hormonal action. However, with enzymes the solution of this problem may be somewhat simpler, and the correlation between chemical structure and biochemical function may be more direct. At least it appears probable that this is the case for the enzymic site. For a minority of low-molecular-weight enzymes that resemble insulin in also being rich in disulfide bonds and hard to denature (ribonuclease, lysozyme, cytochrome c), complete structural elucidation is within our grasp, and may ultimately lead to an understanding of their mode of action. But for the more typical protein-enzymes, this goal is not yet within the field of vision, for methods to deal with proteins of molecular weights of the order of 100,000 are still lacking; furthermore, it is not even known how to attack the problem without breaking most of the hydrogen bonds that represent an integral part of structure and function of this class of proteins. As yet, then, the only approach is to attempt definition of the active site by methods that will not interfere with enzymic activity.

In this talk will be summarized and reviewed several isolated pieces of work, done or in process at the Virus Laboratory in Berkeley and elsewhere, that are concerned with either structural (i.e., amino acid sequence) studies on enzymes and other proteins, or with attempts to characterize an enzymic site directly (i.e., from the middle rather than from the end).

SEQUENCE AND CHAIN-END STUDIES

Tobacco mosaic virus (TMV). The remarkable finding of Harris and Knight ('52, '55) that carboxypeptidase releases 2900 residues of threonine from TMV has now been described in detail and extended to all bonafide strains of TMV that were studied. The conclusion that these are actually the C-terminal residues of about 3000 peptide chains was definitely confirmed when C.-I Niu of this laboratory found a similar number of free threonine residues after hydrazinolysis (Akabori *et al.*, '52; Ohno, '54) of the virus or of its small-molecular protein subunit. In both the enzymic and the chemical studies the cleanness of the reaction, i.e., the absence of other free amino acids, was quite remarkable. When hydrazinolysis was used after enzymic removal of the threonine, alanine was found C-terminal; an additional product which was present only after short periods of heating with hydrazine proved to be prolylalanine. Thus the sequence of three amino acids from the carboxyl end of the peptide chains comprising the virus is now known (Niu and Fraenkel-Conrat, '55) (table 1). Attempts to find amino or imino ends in corresponding number have continued to give negative results (Fraenkel-Conrat and Singer, '54). The present picture of the virus is that of a helical arrangement of about 3000 peptide chain subunits shaped like the figure six, their C-terminal threonine residues pointing outward, and many of their basic groups nearer to the axis of the rod where the nucleic acid appears to be located (Hart, '55).

C-terminal amino acids of various proteins by hydrazinolysis. Ovalbumin is another protein in which amino ends have never been found, although searched for by several groups of workers. On the other hand, C-terminal alanine was claimed on the basis of carboxypeptidase studies and confirmed by hydrazinolysis and by the Schlack and Kumpf thiohydantoin method, before the validity of the enzymic evidence was withdrawn (Steinberg, '52, '53; Turner and Schmerzler, '53; Braunitzer, '54). It was therefore a great surprise to find by hydrazinolysis one residue of C-terminal proline in ovalbumin. This was the case with three different preparations, one of which was kindly supplied by Dr. G. E. Perlmann.

TABLE 1

Advances in elucidation of protein structure

PROTEIN		LOCATION OF AMINO ACIDS		
		N-terminal	Internal	C-terminal
Tobacco mosaic virus (three strains)		None		–Pro–Ala–Thr
Ovalbumin		None		–Val–Ser–Pro
Bovine serum albumin		Asp–Thr–		–Ala
β-Lactoglobulin		Leu– Leu–		–Ileu –Ileu
Chymotrypsinogen		Cys < S–		None
Chymotrypsin	(π)	Ileu–Val–		Ser–Arg
	(δ)	Ileu–Val–		–Leu
		Ala–		–Tyr
	(α)	Ileu–Val–		–Leu
	(All)	Cys < S–		
Crotoxin				
Soluble DNP–crotoxin		Ser–		–(Ser,Phe)–Thr–Asp
Insoluble DNP–crotoxin		None		None
			–Porphyrin–	
Cytochrome c		His–	–Lys–Cys–Ala–Glu–Cys	
		His–	Lys–Glu–Val–Thr–His	

Very recently, two peptides were also isolated after partial hydrazinolysis (seryl-proline and valyl-seryl-proline), which have revealed the amino acid sequence near the C-terminal proline of ovalbumin (table 1).

In bovine serum albumin, a monochain protein-containing N-terminal aspartic acid (Thompson, '54), Niu has now identified alanine as the C-terminal amino acid, and adjacent amino acids are also being isolated in the form of peptides.

β-Lactoglobulin contains N-terminal leucine; in this laboratory two are found, whereas Porter ('48) indicated the presence of three such residues per mole. Carboxypeptidase has yielded data which were interpreted as indicating the presence of C-terminal leucine and histidine (Neurath *et al.*, '54). However, only leucine was found by hydrazinolysis, in an amount indicative of two chains terminating in this amino acid. It would therefore appear probable that histidine occurs as a penultimate amino acid.

Chymotrypsinogen has been regarded as a cyclic protein, until Bettelheim ('55) demonstrated the presence of N-terminal half-cystine. In experiments at the Virus Laboratory, no corresponding C-terminal amino acid could be detected. However, the limitations of the hydrazinolysis method must be clearly recognized. Cystine and cysteine are completely destroyed under the conditions of hydrazinolysis, whereas C-terminal glutamine and asparagine yield monohydrazides that would be removed with the other amino acid hydrazides and thus escape detection. The dicarboxylic acids are recovered in poor yield. Arginine gives ornithine; Locker ('54) noted its disappearance only, and listed it, therefore, with the amino acids for which the method failed.

In attempts at a comparative evaluation of carboxypeptidase and hydrazinolysis, it appears that both methods may fail to reveal certain C-terminal amino acids, although hydrazinolysis has fewer and less-unpredictable failures. Furthermore, the enzyme often releases more than one amino acid, in which case interpretation is largely guesswork. In contrast, hydrazinolysis clearly differentiates the terminal from adjacent amino acids, and may thus supply unambiguous sequential information (see table 1).

Crotoxin. Crotoxin is the crystalline protein from rattlesnake venom carrying the neurotoxic and the hemolytic (lecithinase) activity (Slotta and Fraenkel-Conrat, '38). It has now been possible to fractionate this protein-complex into two very dissimilar components, one a very basic, the other an acidic protein (Fraenkel-Conrat and Singer, '55). Unfortunately, the method of fractionation was of such a nature as to cause complete inactivation of both biological activities. End-group studies indicate that one component has an N-terminal serine residue and C-terminal aspartic acid followed by threonine and serine or phenylalanine (table 1); the other component appears to have no chain ends. It is noteworthy that extensive digestion of the unfractionated crotoxin by carboxypeptidase, causing the release of three amino acid residues per complex-weight (30,000) did not affect the neurotoxic or the lecithinase activity. This is quite in contrast to all chemical agents, since these cause inactivation of the enzyme (Fraenkel-Conrat and Fraenkel-Conrat, '50). It appears that crotoxin is sensitive when attacked in the middle, but not when altered near one chain end. This seems to be a general phenomenon. Most biologically active proteins that have been studied seem to retain their activities after release of one or more amino acids from the carboxyl end; indirect evidence suggests that the amino ends are similarly uninvolved in the active site. It appears that the chain ends, important as they are for all structural work, are of little significance for the biological activities of proteins. The impatient research man is thus tempted to look for short cuts to the heart of the enzyme, the active site, and to postpone the detailed study of the anatomy of its extremities.

STUDIES OF THE ENZYMIC SITE

Amino acid sequences. The first attempt to isolate and chemically characterize the enzymic site is being carried out on cytochrome c by Tuppy and coworkers in Vienna. From partial hydrolyzates, Tuppy has isolated various peptide fragments marked by their attachment to the colored porphyrin ring. This has enabled him to determine the amino acid sequence of a decapeptide containing the two half-cystines that are bound by thioether linkage to the porphyrin (Tuppy and Bodo, '54a, b) (table 1). The isolated porphyrin peptide complex carries some catalytic activity, but even in this case, true enzymic activity appears to be a more macromolecular phenomenon, dependent on the imbedding of much of the porphyrin in the hydrogen-bonded folds of the protein.

Tuppy, in accord with earlier workers, found evidence that histidine played a particular role in the interaction of this peptide with the iron in the prosthetic group. It is of interest, therefore, that two histidine residues have since been claimed to occur in N-terminal position in cytochrome c; one of these terminal histidines is strongly masked and thus presumably coordinated with the iron (Margoliash, '55).

Modification of the protein. The classical approach to the problem has been through chemical modification of the enzyme protein. Methods were therefore devised for the selective attack on only one class of protein groups, and much information was accumulated concerning the occurrence of ''essential'' sulfhydryl, amino, phenolic, and other groups in or near the enzymic site (Herriott, '47; Olcott and Fraenkel-Conrat, '47; Fraenkel-Conrat, '54). This approach has been most fruitfully applied in studies of the role of –SH groups because of the availability of specific, including some reversible, reagents for this group. Unfortunately, the reactions for the other classes of groups are generally less specific and less gentle, and rarely reversible, and thus much of the data obtained by modification techniques is difficult to interpret. The seemingly negative findings that enzymic activity is not affected by chemical modification of most groups of a given type is probably of greater validity than the loss of activity produced by a ''specific reagent.'' Little progress has been made in this field of research in late years. No definite picture of an enzymic site has been proposed on the basis of such work. Yet, any future hypothesis will have to take into account the accumulated findings concerning the effects of varying reagents on biological activity. These studies have revealed a great range in the reactivity of various protein groups, from one protein to the next as well as within one protein molecule. It appears that the most readily reactive are generally not those ''essential'' groups associated with biological activity.

A variation of this technique is based on the ability of the substrate to preserve the activity of certain enzymes in the presence of an inhibiting protein reagent. This approach has led to the suggestion that, in enzymes such as glyceraldehyde phosphate dehydrogenase, the aldehydic substrate and an –SH group of the protein may actually form a hemimercaptal, which would subsequently be oxidized to a thioester. This work, which has been extensively reviewed and discussed (Racker, '54; Boyer and Segal, '54), represents one of the first instances in which a definitive role has been ascribed to an essential thiol group.

Another interesting case is that of rhodanese, the enzyme that catalyzes the detoxification of cyanide to thiocyanate. The active group of this enzyme is believed to be an extraordinarily reactive disulfide bond (Sørbo, '53) that enters into the reaction by combining with thiosulphate; cyanide then splits this bond, regenerating the disulfide bond; thiocyanate and sulfite are the end products.

Modification of substrate. A technique used extensively in attempts to define the active site relies on the chemical modification of the substrate rather than the enzyme. Considerable information concerning the steric and electrochemical requirements of cholinesterases, peptidases, and other enzymes has been accumulated over the years with such methods (Smith, '51; Wilson, '54). This material would represent a suitable subject for review and critical evaluation.

Reaction of esterases with diisopropylfluorophosphate (DFP). Finally, a new method of attack on the enzymic site of at least one class of enzymes has developed out of wartime research on certain organic phosphates, alias the nerve gases. The toxicity of these esters was found to be caused by their reacting in stoichiometric and stable manner with the enzymic site of esterases (Jansen *et al.,* '49, '50; Balls and Jansen, '52). Thus this class of inhibitors bridges the gap between the two lines of approach mentioned in the sections on modification of the protein and substrate. These compounds represent protein reagents specific for the enzymic site, as well as modified substrates, inhibitory not in the customary competitive manner but almost irreversibly through primary bond fixation to the site.

Experiments to determine the mode of attachment of these phosphate esters are actively being pursued by several groups of investigators. There is no doubt that none of the typical polar protein groups can be directly and singly responsible for this binding. No protein other than esterases, no peptides, and no amino acids have been found to react to any extent with alkyl diflurophosphates (DFP) under the conditions of complete inhibition of the enzymes (Balls and Jansen, '52). The inability of even the zymogens (e.g., chymotrypsinogen) or the denatured enzymes to bind the phosphate esters shows quite conclusively that this phenomenon is dependent on the native state of the enzyme, and thus is probably caused by a cooperative reaction involving several amino acid residues held in sterically fixed juxtaposition.

Studies with P^{32} have shown that in acid hydrolyzates the phosphate ester group is attached largely to a serine residue (Schaffer *et al.,* '53). From an enzymic digest, a pentapeptide containing the labeled phosphate was also isolated, in which serine was the only polar amino acid residue (Oosterbaan *et al.,* '55). Yet for the reasons discussed it seems very unlikely that this is the primary site of attachment.

Another set of observations seems of similar if not greater significance. Weil and coworkers ('53) found evidence that photooxidative destruction of one of the two histidine residues of chymotrypsin led to complete inactivation; DFP was no longer bound by this inactivated enzyme. It was further observed by Wagner-Jauregg and Hackley ('53) that histidine and other imidazoles catalyzed the hydrolytic decomposition of DFP. This strongly suggests the formation of imidazole N-isopropyl phosphate as a labile intermediate. In the course of a completely unrelated study, evidence has been found that extremely labile reaction intermediates can be protected and permanently preserved by steric masking within the native protein structure (Fraenkel-Conrat, '55). This suggested that imidazole dialkyl phosphates might, in a similar manner, be stable in the native protein, and that transfer of the phosphate to a serine residue, which also must be in close proximity to and part of the active site, might be a consequence only of denaturation or enzymic unmasking of the crucial histidine residue. This hypothesis is now being experimentally tested in this laboratory by Paul Wigler. It is far too early to describe these experiments or attempt any definite conclusions. But indications are that one histidine residue in native DFP-chymotrypsin is less available for other reactions than it is in the active enzyme.

CONCLUSIONS

One of the key problems in biochemistry is the mechanism of enzyme action. There appear to exist two main paths of research leading toward the elucidation of this problem. One that has only begun to be explored is the complete structural analysis of proteins. Great inroads are being made in this field. It seems probable that within a few years the complete structure will be known for those relatively small protein enzymes that owe their great resistance to many disulfide rather than to hydrogen bond crosslinks.

The more typical enzymes, however, will represent a much greater analytical problem, and even complete elucidation of their amino acid sequences does not signify an understanding of their structure. The enzymic site may well be delineated by protein groups from two or three different peptide chains, and the structure of these proteins seems to be at least as dependent on hydrogen bonds as on primary bonds and amino acid sequences.

With these typical enzymes of high complexity, most research efforts are aimed directly toward the enzymic site. By modification of certain protein groups, and by the use of modified substrates or inhibitory analogs, information concerning the chemical and physical shape of the active site has been accumulated. However, here also progress has been limited by the scarcity of good methods and nobody has yet arrived at a definite representation of the active site of a biocatalytic protein.

The discovery that DFP and similar polyalkyl phosphates inhibit most esterases almost irreversibly by stoichiometric reaction with, or in the vicinity of, their enzymic site has greatly stimulated further work and may yield the key to the active site of this class of enzymes. Experiments with chymotrypsin that implicate a histidine residue have been reviewed, and current work favoring this hypothesis has been briefly outlined. Furthermore, some recent contributions to knowledge of protein structure, obtained at the Virus Laboratory and elsewhere, have been surveyed.

GENERAL DISCUSSION

MOUNTER[1]: Since we have been working on a group of enzymes that split DFP without inhibition, I think there is some justification for trying to add to this fluorophosphate story. These enzymes are esterases and other tissue enzymes which hydrolyze DFP and other organophosphorus quanticholinesterases at a high rate but do not attack nonalkylated phosphate esters. Most of our work has been carried out with a hog kidney enzyme that is activated by Mn^{++} and Co^{++} and whose natural substrates are, I believe, acylated amino acids. There is some evidence, both from *p*H dependence studies and photooxidation, that the active center contains histidine, which is the site of binding of the electrophilic carbon atom of the ester or the phosphorus of DFP. Confirmation of our conclusions that there is a similar action between acyl or dialkylphosphoryl groups with a number of different enzymes and that the site of interaction is the histidine moiety would emphasize the idea that particular structures may repeat in different enzymes, and that these structures are responsible for the binding of specific parts of the substrate.

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[1] L. A. Mounter, University of Virginia School of Medicine.

STEINBERG[2]: My comments are directed to the question of ovalbumin structure, which Dr. Fraenkel-Conrat's findings have thrown into some confusion, I believe. In fact, one might say he has left us with a scrambled egg! His finding of a C-terminal proline in ovalbumin is in disagreement with the results of Turner and Schmerzler and the results of Akabori and his coworkers, who have reported C-terminal alanine. Our own studies have shown that DFP-treated carboxypeptidase does not attack native ovalbumin. However, untreated carboxypeptidase, even though repeatedly recrystallized, releases 0.8–1.05 moles of alanine per mole of ovalbumin and less than 0.3 mole of other amino acids in total. At the suggestion of Dr. Linderstrøm-Lang, we tried to crystallize the ovalbumin from which this alanine had been split. It crystallized readily but in the form of plates rather than the needles characteristic of ovalbumin. We called this new crystalline protein plakalbumin C because of its obvious relation to previously described plakalbumin.

DFP treatment abolishes this alanine-yielding reaction but the addition of low concentrations of subtilisin to the DFP-treated enzyme closely imitates the effect of untreated carboxypeptidase. Apparently, subtilisin makes available to carboxypeptidase a previously masked alanine residue, either by splitting a peptide bond or by partially unfolding the molecule.

Aside from the other chemical end-group studies, Dr. Fraenkel-Conrat's findings and ours would be perfectly compatible on the following basis: If ovalbumin is a cyclic molecule with a tail terminating in proline jutting out from it at some point, carboxypeptidase would not, as we have found, attack it at a significant rate. If subtilisin breaks a bond in the cyclic portion of the molecule it will, in the presence of carboxypeptidase, lead to the release of alanine alone, as we have observed.

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[2] Daniel Steinberg, National Heart Institute.

WEIL[3]: A reinvestigation of the tryptic conversion of chymotrypsinogen to chymotrypsin might be required in the light of our recent findings, which have shown that crystalline trypsin is enzymically heterogeneous.

I should also like to mention the possible importance of histidine to catalytic activity observed by Egan and coworkers. These investigators have found that if DP-chymotrypsin was photooxidized, only 1 mole of histidine of the total of 2 was susceptible to oxidation, in contrast to the native enzyme, where ultimately both units have undergone photooxidation. These findings strongly suggest that the attachment of DFP to chymotrypsin took place on the histidine molecule, and thus supply added support to the significance of the imidazole ring to the catalytic activity of chymotrypsin.

FRAENKEL-CONRAT: In regard to Dr. Steinberg's remarks, I only wish to reiterate that we believe we are studying a different part of the egg albumin molecule to that which was attacked by enzymes used in his work at the Carlsberg Laboratory. Our only disagreement is with Akabori's hydrazinolysis data, which is being reinvestigated through an exchange of samples; it appears probable that proline was mistaken for alanine. There is no doubt concerning our finding of a C-terminal valine-serine-proline peptide in all preparations of ovalbumin.

Dr. Weil's comments are very interesting. I was glad to hear that another approach has also indicated that a histidine group may be involved in the active center, and in the DFP binding, of chymotrypsin.

[3] Leopold Weil, USDA, Eastern Utilization Research Branch.

BELL[4] : Throughout his talk Dr. Fraenkel-Conrat has urged great caution in interpretation of the results of various methods. The utility of any of these methods can be established only by a study of peptides whose structures are known by other means. In this connection, our preliminary studies on known peptides, and finally on ACTH, whose structure is now established by other means, appears pertinent.

In our hands, the hydrazinolysis method of Akabori led to peptide formation during the reaction. In addition to the C-terminal amino acid, peptides were isolated also from the reaction mixture. Structure determination of one of these peptides subsequently showed that it originated from the central portion of the ACTH molecule. It is our opinion that hydrolysis may occur during the hydrazinolysis unless all bound water is first removed from the peptide and the reagents. With certain peptides, this was not possible even with a 10^{-6}-mm vacuum applied for 96 hours at 100° C.

We found carboxypeptidase extremely useful, but in certain cases this method failed. For example, two of the active pepsin digest products of β-corticotropin have the C-terminal sequences

. . . Val-Tyr-Pro-Asp-Gly-Ala-Glu-Asp-Glu-$(NH_2)\cdot$OH
. . . Val-Tyr-Pro-Asp-Gly-Ala-Glu$\cdot$OH.

In the first case, carboxypeptidase would remove glutamine only; in the second, there was no digestion even with very high enzyme levels. This was also observed when the smaller peptides corresponding to the sequences shown were used. It would, therefore, appear that this method may fail when two or more acidic groups are near the point of digestion.

A few comments on other methods mentioned by Dr. Fraenkel-Conrat: Our studies showed that the carbodiimide method, recommended by Dr. Kenner of Todd's laboratories, is not of general application. The steric efforts of the penultimate-R group seem to control the reaction. The widely used DNP method also sometimes leads to confusion. In the N-terminal sequence of β-corticotropin,

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[4] P. H. Bell, American Cyanamid Company.

H·Ser-Tyr-Ser-Met . . .,

the reaction with DNFB ties up essentially all of the serine, although phenylisothiocyanate does not react with the second serine. Our data suggest that the tyrosylseryl bond is labile under the alkaline conditions of the DNFB reaction. This split would uncover the second serine for reaction.

I have been thinking about the problem of peptide chain branching mentioned by Dr. Haurowitz. In any structure containing aspartic and glutamic acid residues, one is always faced with the possibility of β and γ linkages. Our preliminary studies with model peptides suggest that hydrazinolysis may be useful in solving this problem. If branching occurs on the α,β or α,γ carboxyls, dihydrazides should be formed. On the other hand, if the peptide chains are not branched, two monohydrazides of each amino acid are possible. Preliminary studies suggest that the three possible hydrazides of each of these amino acids may be separated by paper chromatography.

Fraenkel-Conrat: Concerning Dr. Bell's finding of a non-C-terminal peptide after hydrazinolysis, I can only say that we have been concerned about possible effects of traces of water. We have dried the proteins and hydrazine vigorously, but we actually get no appearance of spurious C-terminal amino acids if 10% of water is added to the hydrazinolysis. The remarkable cleanness of our chromatograms, which show no traces of other than the terminal DNP-amino acid, with so many proteins gives reason to believe that we are on safe ground.

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THE EFFECT OF OPTICAL CONFIGURATION OF AMINO ACID RESIDUES ON THE PHYSICAL PROPERTIES OF PEPTIDES

ERIC ELLENBOGEN
Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

TWO FIGURES

Advances in the preparation of optically pure amino acids, as well as improved methods of synthesis, have made available many interesting peptides of known optical configuration. A number of years ago a study was begun on the effect of optical configuration on the dissociation constants of the ionizable groups and on the infrared spectra of peptides. In all cases, the optically "pure" peptides, composed only of L amino acid residues, were compared with optically "mixed" ones in which one L residue at a time was replaced with its D isomer.

Optical configuration may be presumed not to influence the physical properties in those compounds containing but a single asymmetric carbon atom. This has been shown to be true for the pK' values of the carboxyl and amino groups in the following compounds (Ellenbogen, '52):

		pK_1'	pK_2'
Glycylalanine	(L)	3.17	8.23
Glycylalanine	(D)	3.16	8.24
Alanylglycine	(L)	3.17	8.18
Alanylglycine	(D)	3.20	8.19

The measurements were carried out at 25° C. at constant ionic strength of 0.100, the Beckman model G pH meter being used. In view of the experimental error of $\pm$ 0.03 pK' units, these ionization constants may be considered to be identical, and independent of the order in which the two amino acids are linked. The infrared spectra of these compounds, however, permit differentiation between glycylalanine and alanylglycine, but they do not show any difference between the L and D residues in each pair.

As soon as more than one optically active amino acid residue is introduced in the peptides, differences appear also in the ionization constants. A comparison may be made, for example, of all possible isomeric peptides of trialanine:

Alanylalanylalanine	(LLL)	pK_1'	3.39	pK_2'	8.03
	(LLD)		3.37		8.05
	(LDL)		3.31		8.13
	(DLL)		3.37		8.06
	(DDD)		3.39		8.06

The infrared spectra of the (LLL) and (DDD) compounds are identical, but marked differences are observed in the optically mixed peptides. Similar results have been observed on all such groups of peptides studied thus far; in those containing lysine, an effect is observed even on the pK_3', which belongs to the ε-amino group.

TABLE 1

Dissociation constants of the alanylalanines

TEMP.	pK_1' (COOH)		pK_2' (NH_2)	
	(LL,DD)	(LD,DL)	(LL,DD)	(LD,DL)
°C.				
8.7	3.22	3.05	8.57	8.70
25.0	3.30	3.12	8.14	8.30
36.8	3.28	3.14	7.93	8.03

For further study of this phenomenon, investigations were made of the simplest peptide containing two asymmetric carbon atoms, alanylalanine, whose four isomers were prepared. These were titrated at three different temperatures, in order to estimate standard thermodynamic quantities associated with the ionization of the two functional groups. An improved *p*H meter, Beckman's model GS, made possible the determination of pK' values with a precision of ± 0.01 pK' units. The results of these measurements are shown in table 1. From these constants the standard changes in entropy, enthalpy, free energy, and heat capacity were computed. They were calculated according to the method of Harned and Robinson ('40), based on a linear increase with temperature of the change in heat capacity. Since there were measurements at three temperatures only, another set of derived thermodynamic values was calculated, on the assumption that over the temperature range studied, the change in heat capacity remains constant (H. Frank, personal communication). Since both sets of equations yield results identical within the experimental error, only those derived from Harned and Robinson's treatment are listed (table 2).

TABLE 2

Thermodynamics of the ionization of the alanylalanines

PEPTIDE →		(LL,DD) Temperature (°C.)		(LD,DL) Temperature (°C.)	
		25	37	25	37
COOH	ΔF°	4,498	4,655	4,250	4,450
	ΔH°	—369	1,905	—1,111	—298
	ΔC_p°	175.2	182.3	66.4	69.1
	ΔS°	—16.1	—8.9	—18.0	—15.3
NH_2	ΔF°	11,079	11,224	11,299	11,364
	ΔH°	8,603	6,321	9,557	9,773
	ΔC_p°	—186.4	—193.9	17.7	18.4
	ΔS°	—8.3	—15.8	—5.8	—5.1

Estimated experimental error: ΔF° : ± 25 cal/mole
ΔH° : ± 100 cal/mole
ΔC_p° : ± 12 cal/mole/degree
ΔS° : ± 0.3 e.u./mole

In all measurements, the results obtained for the (LL) peptide were identical with those obtained from the (DD), and those obtained from the (LD) were identical with those from (DL). Since (LL) is the mirror image of (DD), and since (LD) is the mirror image of (DL), this is not surprising. Here again, the infrared spectra of the pure peptides are identical, but markedly different from those of the mixed peptides, which in turn are identical. In table 3 are listed those frequencies for the four peptides, for which a tentative assignment is proposed.

A number of interesting deductions may be made on the basis of these results. From molecular models, a rough decision may be made as to whether the peptide linkage is in the *cis* or *trans* configuration. It is assumed that the bond dis-

TABLE 3

Tentative frequency assignment of the alanylalanines [a]

ASSIGNMENT	(LL,DD)	INTENSITY	(LD,DL)	INTENSITY
	cm⁻¹		*cm⁻¹*	
NH stretch, H bonded	3208	Medium	3355	Medium
	3049	Strong	3177	Weak
CH stretch	2967	Strong	2996	Medium
	2932	Strong	2979	Medium
			2927	Medium
			2835	Medium
NH stretch from NH_3 +			2082	Weak
CO, CN stretch from peptide resonance	1610	Strong	1625	Strong
	1555	Medium	1565	Strong
CO stretch, H bonded	1685	Medium	1670	Strong
CN stretch and/or NH deformation	1528	Strong	1517	Strong
	1287	Medium	1280	Medium
	1262	Medium		
CH deformation	1460	Medium	1450	Strong
	1407	Strong	1414	Strong
CNC stretch	1008	Weak	993	Medium
			776	Medium
CH, CH_3 rock	956	Medium	946	Medium
	912	Weak	917	Weak
CH, NH rock	732	Medium	760	Weak
	680	Weak	721	Weak
			688	Medium

[a] From Ellenbogen, '56.

Grateful acknowledgment is hereby extended to Dr. Foil A. Miller, Mellon Institute, Pittsburgh, who determined these frequencies.

tances and bond angles are those established for the extended polypeptide chain (Corey and Pauling, '53), but the amino hydrogen is placed *cis* to the carbonyl oxygen. In a model, the striking feature of this arrangement is the close approach of the α hydrogens (belonging to the asymmetric carbon atoms). On the basis of Lassettre and Dean's treatment ('48) of hydrogen-hydrogen repulsions, it is estimated that the *trans* configuration is approximately 4000 cal per mole more stable than the *cis,* even in the optically mixed peptides. From a comparison of the (LL,DD) and (LD,DL) pairs in the *trans* configuration, it is estimated that the (LL,DD) pair is about 240 cal per mole more stable than the (LD,DL) pair, based again on hydrogen-hydrogen repulsions involving only the α hydrogens, the interaction due to methyl groups being neglected. In a qualitative sense, this could account for the difference in the free energies of ionization of the functional groups, which is of that order of magnitude.

The validity of this treatment may be tested by examining the infrared spectra of the two pairs of peptides. From the models it may be assumed that the infrared spectra of the (LD,DL) pair of peptides should show bands belonging to the skeletal C-N-C vibration, and shifted to frequencies lower than expected for (LL,DD). Owing to the proximity of the two methyl groups, shifts in the C-H vibrations would also be expected, as well as some additional C-H frequencies.

Provided that the frequency assignment in table 3 is valid, the C-H stretching vibrations produce two strong bands in the pure, and four medium bands in the mixed peptides. Only a weak band corresponding to the skeletal C-N-C stretching vibration shows up in the pure, whereas two medium bands at lower frequencies are assigned to this mode in the mixed peptides.

Molecular models furnish other information in addition to that apparently substantiated by infrared spectra. The arrangement of the atoms and the relative position of the ionizable groups on each end of the peptide, also give some clues as to phenomena associated with the ionization processes. Of particular interest is an explanation of the entropy changes associated with the two types of ionization, namely, the removal of a proton from an uncharged group (giving rise to a dipolar ion), followed by the removal of a second proton from a positively charged group (giving rise to an anion). The creation of two charges on opposite ends of the same molecule might be expected to cause a folding that would involve a loss of entropy. The second ionization creates an anion, and the consequent removal of the attractive forces between two charges of opposite sign on the two ends of the peptide would be expected to allow the molecule to unfold. The straightforward isoelectric reaction $^{+}H_3NCH(CH_3)$-$CONHCH(CH_3)COOH + OH^{-} \rightleftharpoons H_2NCH(CH_3)CONHCH$-$(CH_3)COO^{-} + H_3O^{+}$ should not involve any appreciable change in the heat capacity, and it may be postulated that the simple mechanism of folding would contribute an entropy term which is to be added to the entropy term caused by ionization. A two-term entropy contribution seems to be reasonable for mixed peptides. This simple picture does not hold true, however, for the pure peptides. In the mixed peptides, the values for $\Delta C_p°$ are small, and essentially identical within experimental error. This is not so with the pure peptides, where they are large and of opposite sign. This might be caused by at least one additional phenomenon accompanying the two ionization processes, and from studies on the differential entropy of dilution of amino acids (Robinson, '46), it is reasonable to assume that a change in the crystallinity of the water molecules surrounding the polar centers of the peptides may contribute another entropy term. A proposed breakdown is summarized in table 4, in which it is assumed that the standard entropy changes of ionization for peptides are of the same order of magnitude as those for amino acids (Cohn and Edsall, '43). Should this be correct, even as only an approximation, it is attractive because it might be interpreted as meaning that, at physiological temperatures, as small a peptide as optically "pure" alanylalanine (LL,DD) begins to assume a type of folding leading to the helical polypeptide configuration. An increased order of water molecules surrounding the peptide, or some other phenomenon leading to entropy changes, would have to be postulated also from the very qualitative observation that the pure peptides cannot fold as easily as the mixed ones. In the mixed peptides, the two methyl groups leave the peptide backbone on the same side, thus allowing sufficient freedom to fold in a direction away from the methyl groups, but in the pure peptides the two methyl groups are *trans* to each other, and therefore they constitute some obstacle to any folding process that retains the planarity of the peptide linkage.

TABLE 4

Entropy summation for the ionizations of the alanylalanines

PEPTIDE →	(LL,DD) Temperature (°C.)		(LD,DL) Temperature (°C.)	
	25	37	25	37
Cation-dipolar ion due to:				
H_2O	—3	—1		
Folding	—4	—1	—10	—7
Ionization	—8	—8	—8	—8
Total	—15	—10	—18	—15
Dipolar ion-anion due to:				
H_2O	0	—2		
Folding	0	—4	+3	+4
Ionization	—9	—9	—9	—9
Total	—9	—15	—6	—5

Other peptide pairs now being studied show similar interesting properties and apparent high orders of spatial specificity. Of the many pairs, the glutamylglutamic acids are most interesting. At present, the infrared spectra of the (LL) and (LD) α- and γ-glutamylglutamic acids are the only ones available (fig. 1, 2). Of interest are the rather diffuse spectra for the α-LD and γ-LL peptides below 1300 cm^{-1}. All four peptides show intense bands around 1400 cm^{-1} which are probably caused by unionized COOH, known to be present in all of them. The hydrogen-bonded N-H stretching region

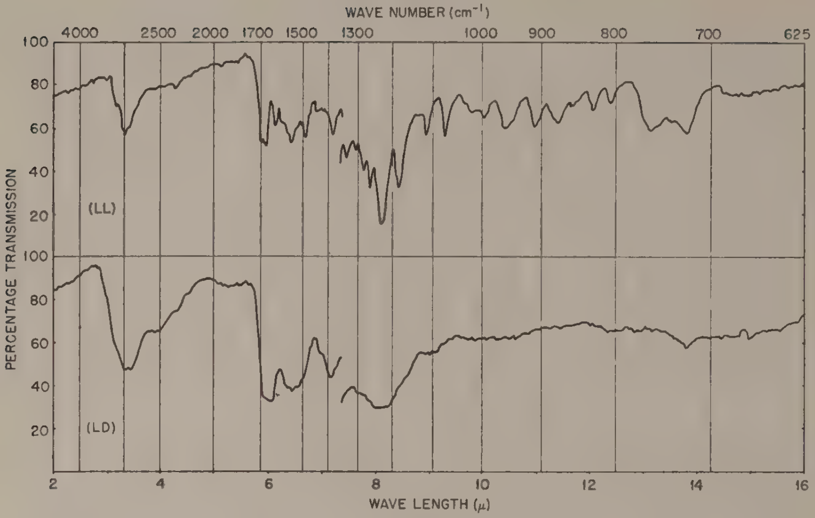

Fig. 1 Infrared spectra of α-glutamylglutamic acids in Fluorolube and Nujol mulls. Top: (γ)H Glu-Glu OH(LL); bottom: (γ)H Glu-Glu OH(LD).

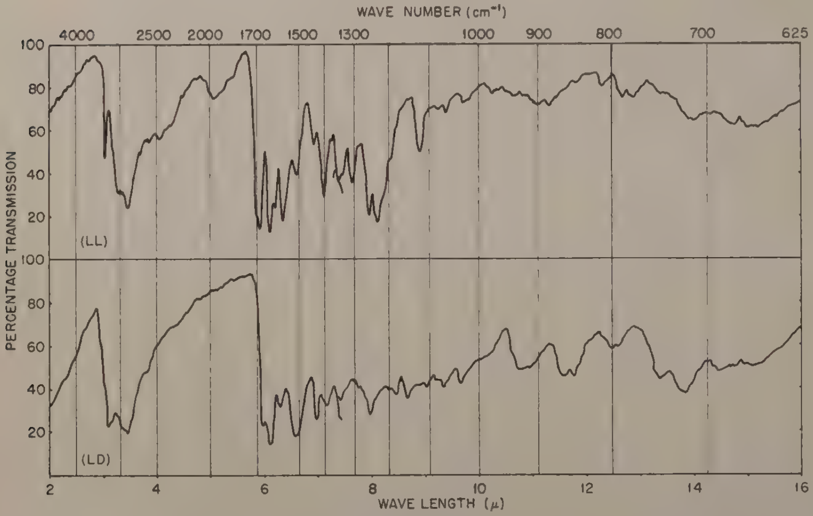

Fig. 2 Infrared spectra of γ-glutamylglutamic acids in Fluorolube and Nujol mulls. Top: (γ)H Glu-Glu OH(LL); bottom: (γ)H Glu-Glu OH(LD).

near 3200 cm^{-1} is not clearly separated from the C-H stretching regions around 2980 cm^{-1}. In the (LL) peptide, C-N-C stretching frequencies occur at 910 and 995 cm^{-1}. Tentative frequency assignments were not attempted because of the complexity of the spectra due to the mutual interactions between the many groups. On the whole, these spectra begin to resemble infrared spectra of proteins.

In conclusion, it should be pointed out that a distinction probably cannot be made between the α and β configurations of polypeptides from infrared measurements. Such a discriminating feature was deduced by Ambrose and Elliott ('51) who ascribed the 1585 and 1550 cm^{-1} bands to the N-H stretching frequency and the 1658 cm^{-1} band to C-O stretching frequencies in the α configuration. As the polypeptide assumes the β configuration, these frequencies shift to 1570, 1527, and 1629 cm^{-1}, respectively. Many of these patterns are seen in the spectra of all small peptides, and it was concluded that Elliott and Ambrose's observation could not be considered a specific property of either the coiled or extended polypeptide configuration.

GENERAL DISCUSSION

SOODAK[1]: You said that in the infrared with the LD-glutamylglutamic acid you get a spectrum that looks like that of a protein. Maybe in proteins there might be an LD-amino acid. Would you like to comment on that?

ELLENBOGEN: I believe that the resemblance of some of these spectra to those observed with proteins is fortuitous. In glutamylglutamic acid (LD) the large amount of mutual interaction between the various groups probably accounts for the diffuse infrared spectra. It is not possible to establish, on the basis of these spectra, whether D amino acid residues occur in proteins, although an occasional D amino acid residue has many attractive possibilities.

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[1] M. Soodak, Massachusetts General Hospital, Boston.

Edsall [2]: Dr. Ellenbogen was kind enough to send us samples of his LD- and LL-alanylalanine, and Dr. David Garfinkel in our laboratory has studied their Raman spectra, both in the isoelectric form and in the form of their hydrochlorides. In the latter case, the Raman spectra arise only from the cation of the peptide. I shall not go into the numerous details, except to say that the Raman and the infrared spectra seem to agree reasonably well. However, the Raman spectra of the LL and the LD compounds seem to be much more nearly alike than the infrared spectra. This is probably to be expected since the Raman spectra were recorded from an aqueous solution, whereas the infrared spectra were in the crystalline state. There would be much more difference between molecular interactions, in the LL and the LD molecules, when they are packed close together in the crystal than when they are separate from one another and surrounded by water molecules.

I think the differences observed by Dr. Ellenbogen in the *pK* values are extremely interesting, and I hope that further work will be done on the theoretical side in calculating the possible molecular configurations and the distances between the ionizing groups in the LD,DL and the LL configurations. Preferably, studies should be done at several different ionic strengths, in order that ionic strength might be extrapolated to zero. It would also be important to know the temperature coefficients of the *pK* values.

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[2] J. T. Edsall, Harvard University.

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CONFIGURATIONS OF POLYPEPTIDE CHAINS AND PROTEIN MOLECULES

JOHN T. EDSALL
Biological Laboratories, Harvard University
Cambridge, Massachusetts

ELEVEN FIGURES

Protein structure may be considered on at least three different levels of organization. First, there is the problem of structure in terms of the classical approach of the organic chemist. For a protein, this involves the determination of the number of peptide chains per molecule, the sequence of amino acid residues in each chain, and the nature and position of the cross links holding the chains together. The second level of inquiry involves the spatial configuration of the individual peptide chains, or chain segments; they may be wound in helical coils, or more or less fully extended, or arranged in specific configurations in local regions, depending on the geometry of the molecule, the intramolecular forces, and the interactions between the protein molecule and the other molecules that surround it. If the surroundings change, the configuration may change also; thus in a medium such as concentrated urea, or in acid or alkaline solution where the acid or basic side chains may acquire a net charge and repel each other, a tightly coiled helix may unfold into a more extended and more random coil. Finally, at the third level of organization, which is closely related to the second, the formation of folds and loops in the peptide chains, and the manner in which the constituent chains pack together to form the whole molecule, must be considered. It may not be possible to answer this question in terms of a single, uniquely defined configuration, even for a protein molecule in the undenatured state.

On a limited scale, many of the amino acid side chains presumably possess some freedom of movement; but there may also be larger segments of the molecule that possess considerable flexibility and can change their orientation relatively to the rest of the structure around their points of attachment. In any case, all evidence indicates a high degree of internal organization; this may involve a considerable number of cross linkages arising from hydrogen bonding between side chains, as, for instance, between a hydroxyl group of a tyrosyl residue and an ionized carboxyl group of an aspartyl or glutamyl residue. Also, the van der Waals attractions between nonpolar side chains may play an important part in maintaining molecular cohesion. The importance of these different factors may differ greatly from one protein to another; even on the basis of present dim and imperfect knowledge, it appears certain that proteins vary widely from one another in their structural patterns.

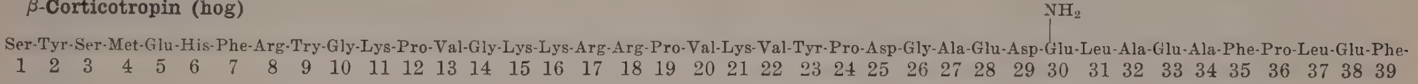
SEQUENCES IN PEPTIDE CHAINS

The state of knowledge at the first level of organization — the sequence of amino acid residues in peptide chains, and the cross linkages of the chains — will be discussed only briefly here, and no attempt will be made to discuss the methods by which the knowledge of these sequences was achieved. The greatest triumph in the field is, of course, the determination of the structure of three species of insulin molecules — those of cattle, sheep, and hog — by Sanger and his collaborators (Sanger and Tuppy, '51; Sanger and Thompson, '53; Sanger *et al.*, '54; Brown *et al.*, '55). Almost equally remarkable is the determination of the structure of hog β-corticotropin (Bell, '54; Howard *et al.*, '55) and of sheep α-corticotropin (Li *et al.*, '55). These are even longer peptide chains than either of the two chains in insulin, but the structure problem is simpler, since each of the corticotropins is a single chain, with no disulfide cross links or sulfhydryl groups. There are of course many other more fragmentary amino acid residue sequence determinations, notably in ribonuclease, which is discussed

TABLE 1

Some major chain sequences already determined

β-Corticotropin (hog)



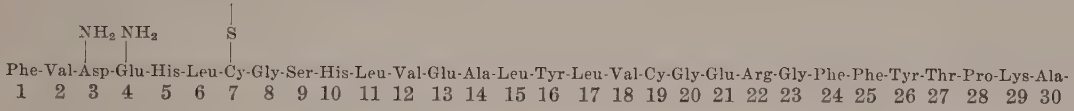
$$\text{Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asp-Gly-Ala-Glu-Asp-}\overset{\mathrm{NH_2}}{\underset{|}{}}\!\!\text{Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-}$$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

α-Corticotropin (sheep)

Sequence same as for β-corticotropin with the following exceptions: Ala-Gly-Glu-Asp (25 26 27 28) and Ala-Ser (31 32). Residue 30 may be glutamic acid in the sheep hormone, rather than glutamine, as reported for the hog hormone.

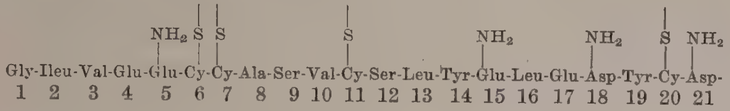
Hog, sheep, and beef insulin, B chain



$$\text{Phe-Val-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Asp}}}\text{-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Glu}}}\text{-His-Leu-}\overset{\overset{|}{\mathrm{S}}}{\overset{|}{\text{Cy}}}\text{-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cy-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-}$$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Beef insulin, A chain



$$\text{Gly-Ileu-Val-Glu-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Glu}}}\text{-}\overset{\overset{|}{\mathrm{S}}}{\overset{|}{\text{Cy}}}\text{-}\overset{\overset{|}{\mathrm{S}}}{\overset{|}{\text{Cy}}}\text{-Ala-Ser-Val-}\overset{\overset{|}{\mathrm{S}}}{\overset{|}{\text{Cy}}}\text{-Ser-Leu-Tyr-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Glu}}}\text{-Leu-Glu-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Asp}}}\text{-Tyr-}\overset{\overset{|}{\mathrm{S}}}{\overset{|}{\text{Cy}}}\text{-}\overset{\mathrm{NH_2}}{\overset{|}{\text{Asp}}}\text{-}$$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Hog insulin, A chain

Sequence same as in beef insulin with the following exception: Thr-Ser-Ileu- (8 9 10)

Sheep insulin, A chain

Sequence same as in beef insulin with the following exception: Ala-Gly-Val- (8 9 10) later, but the ones mentioned are outstanding. These are presented in table 1 and are given in the abbreviated notation for the amino acid residues developed by the late Dr. Erwin Brand (Brand and Edsall, '47).

Figure 1 shows the system of cross linking disulfide bonds found in the insulin molecules. This contains two interchain disulfide links between the A and B chains, and one twenty-atom ring within the A chain, which is closed by a disulfide link. This is located between residues 6 and 11, the residues by convention being always counted from the end that contains a free terminal α-amino group. This ring is of the same type as that found in the small peptide hormones, of the posterior pituitary, oxytocin and vasopressin, by du Vigneaud and his associates ('53, '54). The sequence of peptide linkages, α-carbon atoms, and disulfide linkages is the same in all these structures, although the nature of the side chains is entirely different in insulin from that of the pituitary hormones (fig. 2). It is probable that the actual spatial configuration is also different; the problem of the spatial arrangement of the insulin molecules is considered later.

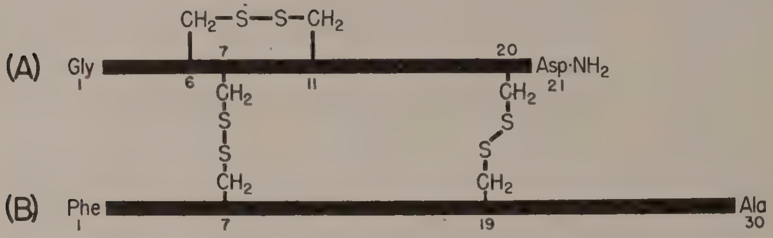


Fig. 1 Diagrammatic representation of the structure of insulin according to Sanger. The cross linkage between the A and B chains by two disulfide links is shown; the disulfide loop in the A chain, between residues 6 and 11, is also shown. Residues are numbered in each chain, beginning at the end containing a free α-amino group. Details of the A and B chain sequences are given in the text.

In any case, it is most remarkable that the species differences between the three insulins depend entirely on variations in the residues 8, 9, and 10 of the A chain, within this intrachain loop closed by the —S—S— bridge. The rest of the A chain, and all of the B chain, is identical for all three species. One might suspect that this particular loop is of crucial importance in the biological function of insulin; it will be interesting to know whether, if this particular disulfide linkage is broken and with no other change in the molecule, the hormone activity of insulin disappears. Yet it is just in this particular section of the molecule, which is probably of crucial importance, that variation of pattern is permitted; the rest of the molecule appears to be tailored to very precise specifications. This represents the first glimpse of what is meant by species specificity in proteins, and the results are tantalizing and unexpected. For instance, in pig insulin, residues A8 and A9 are two hydroxyamino acid groups — threonyl and seryl; whereas in sheep insulin, they are the nonpolar residues alanyl and glycyl. Thus the possibilities for hydrogen bonding involving the side chains in this particular section of the molecule are much greater for the former than for the latter; yet no difference in physiological activity of the two molecules is yet known. The

SUBSTANCE	NATURE OF ATTACHED RESIDUE OR RESIDUES AT POSITION:					
	X	R_1	R_2	R_3	R_4	Y
Beef insulin	....Glu-NH_2	Cy·S-	Ala	Ser	Val	Ser-Leu-Tyr....
Pig insulin	....Glu-NH_2	Cy·S-	Thr	Ser	Ileu	Ser-Leu-Tyr....
Sheep insulin	....Glu-NH_2	Cy·S-	Ala	Gly	Val	Ser-Leu-Tyr....
Oxytocin	H	Tyr	Ileu	Glu·NH_2	Asp·NH_2	Pro-Leu-Gly·NH_2
Arginine vasopressin	H	Tyr	Phe	Glu·NH_2	Asp·NH_2	Pro-Arg-Gly·NH_2
Lysine vasopressin	H	Tyr	Phe	Glu·NH_2	Asp·NH_2	Pro-Lys-Gly·NH_2

Note that residues R_1, R_2, R_3, and R_4 in this figure correspond to residues 7, 8, 9, and 10 in the A chain of insulin in table 1.

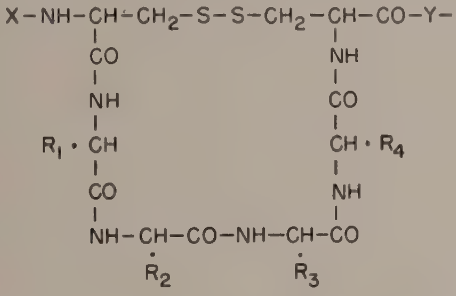


Fig. 2 The peptide-disulfide ring structure of oxytocin, vasopressin, and insulin.

existing bioassay methods are indeed somewhat crude, and further refinements may reveal species differences not yet detected.

It certainly seems too early to interpret the amino acid residue sequence patterns. From the limited data at present available, it appears quite possible that almost any amino acid residue may be placed adjoining any other; nor has any particular evidence for long-range order in the sequences emerged. Indeed, such order, if it exists, is more likely to be found in the larger protein molecules not yet investigated. However, insofar as a trend is detectable, it is for the grouping together of similar types of residues, either polar or nonpolar. The structure of β-corticotropin is particularly notable; all the nine positively charged residues, including the N-terminal ammonium group of serine (residue 1) lie between positions 1 and 21. The sequence of four positively charged residues (Lys.Lys.Arg.Arg) in positions 15–18 is particularly striking. In contrast, all but one of the seven negatively charged groups, including the C-terminal phenylalanine, are located at the other end of the chain, in positions between 25 and 39. Positions 1, 2, and 3 are all occupied by hydroxyamino acid side chains, aliphatic or aromatic. The insulins contain the aromatic sequence Phe.Phe.Tyr. in residues 24–26 of the B chain. The peptide, Ala.Gly.Val.Asp.Ala.Ala, derived from the conversion of ovalbumin to plakalbumin (Ottesen and Villee, '51), contains four nonpolar residues out of six, not including the glycyl side chain; and ribonuclease (R. R. Redfield, personal communication) contains the nonpolar sequence Ala.Ala.Ala near the amino end of its single peptide chain. Earlier speculations, which suggested that the preferred sequences in peptide chains would consist predominantly of alternating polar and nonpolar side chains, appear to be almost the opposite of the truth. The truth, however, is certainly not simple, and the sequences hitherto observed in a few rather small molecules may not be representative of proteins in general.

The grouping together of clusters of amino acid residues of the same sign of charge certainly increases the electrostatic free energy of the molecule; such a structure is obviously less stable in this respect than an arrangement in which the positively and negatively charged groups alternate along the chain. Since nature nevertheless produces at least some — and possibly many — structures of the former type, they may be built in this fashion because they have a job to do. The clustering together of nonpolar side chains, on the other hand, should be a stabilizing influence, not only because of the van der Waals attractions between such residues, but also because the separation of adjacent side chains from one another would involve the filling of the intervening space with water molecules. Hydrogen bonds between the water molecules must be broken to do this, and this would involve the expenditure of a considerable amount of energy (cf. discussion by Kauzmann, '54). Another example of clustering of similar side chain groups has been found for the six sulfhydryl groups of horse hemoglobin, so important for the oxygen affinity of the molecule (Riggs, '52), which appear to be grouped together in two clusters of three each (Ingram, '55). Two of each set of three are readily titratable in the native molecule, and they are close enough together to be linked through a single mercury atom; the third member of each of the two sets is titratable only after the molecule has been denatured. However, until more is known about the actual spatial arrangements it would probably be useless to pursue this line of speculation further.

DISULFIDE AND PHOSPHATE CROSS LINKAGES

Disulfide cross links are undoubtedly the chief covalent bonds involved in the cross linking of peptide chains in proteins. They may join different peptide chains; or they may cross-link different portions of the same peptide chain, thus forming loops. Both possibilities are realized in insulin. In ribonuclease, there are four disulfide links per molecule, and only a single peptide chain with 126 links (Anfinsen *et al.*, '54; Hirs *et al.*, '54); therefore, only the internal loops are involved here. In serum albumin the state of affairs appears to be similar, but on a larger scale; there are fifteen or sixteen disulfide links per molecule, depending on the exact molecular weight assumed, but there appears to be only a single peptide chain. The structure of albumin will be further discussed.

The spatial configuration of the disulfide link is important. Calvin ('54) has considered the question in some detail. The —S—S— bond distance is near 2.04 A in simple dialkyl disulfides; the —C—S— distance is 1.81 A and the —S—S—C— bond angle near 107°. Calvin gives the dihedral angle as nearly 90°; in the group $C_1—S_1—S_2—C_2$, this is the angle between the plane defined by the atoms C_1, S_1, and S_2 and the plane defined by S_1, S_2, and C_2. Moreover, Calvin concludes from thermodynamic data on simple disulfides that the resistance to rotation out of this configuration appears to be very strong; the form in which C_1, S_1, S_2, and C_2 are all coplanar would, by his estimate, be increased in energy by 10–20 kcal/mole, as compared with the stable configuration with dihedral angle of 90°. Thus the completely coplanar configuration would be very improbable, and the —S—S— bond should be almost as rigid as a double bond in maintaining its preferred configuration. Since, however, Yakel and Hughes ('54) have found a dihedral angle of 101 ± 2° for the disulfide group in N,N′-diglycyl-L-cystine, there appears to be no great resistance to rotation of the dihedral angle by 10°, or somewhat more, away from the presumably preferred value of 90°.

The work of Perlmann ('54a, b, '55) has shown that, in some proteins at least, phosphate diester or pyrophosphate cross linkages play an important part in structure. Some phosphate ester groups in proteins are simply primary esters, usually of hydroxyamino acid residues. These, of course, are not involved in cross links. Other linkages, however, are attacked by specific phosphodiesterases, and appear to be of the type shown in figure 3.

In the link illustrated, both amino acid residues are seryl, but threonyl residues may also be involved. Other phosphodiesterases attack links of the —N—P—O— type, involving a nitrogenous group on one of the amino acid chains. The evidence for the existence of these and other cross linkages involving phosphate is presented by G. E. Perlmann in the General Discussion following this paper.

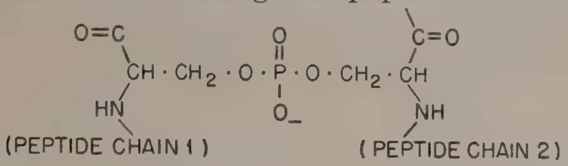


Figure 3

CROSS LINKS INVOLVING HYDROGEN BONDS BETWEEN SIDE CHAINS

Cross links caused by hydrogen bonding must certainly be of importance in stabilizing some protein structures. Those most clearly identified at the present time involve the hydroxyl groups of the tyrosine residues as proton donors. The nature of the proton acceptor groups is less clear, but they may be ionized carboxyl groups of glutamic or aspartic acid residues (fig. 4).

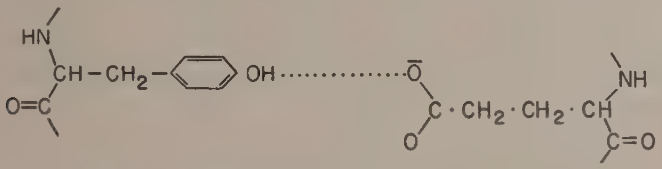


Figure 4

The simplest bond of this type is the intramolecular bond in salicylic acid (fig. 5), when the carboxyl group is ionized. This bond profoundly affects the ionization of the hydroxyl group, which usually has a pK value near 10, characteristic of the simple phenols, of the hydroxyl groups in tyrosine and tyrosine peptides, and of the *m*- and *p*-hydroxybenzoic acids. In salicylic acid, however, the pK value is displaced to approximately 13 (Larsson, '29), owing to the hydrogen bonding. Crammer and Neuberger ('43) in a classical investigation of ovalbumin, showed, by ultraviolet absorption measurements

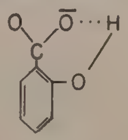


Figure 5

in the region 270–295 mμ, that the tyrosine hydroxyl groups of this protein were not free to ionize until a very alkaline *p*H, above 12, was attained. Then ionization occurred, apparently quite suddenly and in a manner involving irreversible changes in the protein. Other evidence showed that denaturation had occurred, and the ultraviolet absorption indicated that the tyrosine hydroxyls subsequently remained ionized, even at *p*H values considerably below 12, and behaved as if they were free of the attachments that bound them in the native protein. Just how important these bonds are in maintaining the native structure is hard to say; there may be other attachments broken when the molecule is thus denatured that are not so readily detected by spectroscopic measurements but may be as important as the bonds involving tyrosine, or more so. However, it seems probable that the tyrosine groups play a really important part in maintaining the native structure. Apparently, the only other protein yet carefully studied in which the tyrosine hydroxyls are similarly bound is ribonuclease (Shugar, '52; Tanford *et al.*, '55). In this case, it appears that probably half the tyrosine groups — that is, three of the six present in the molecule (Hirs *et al.*, '54) — are involved in hydrogen bonding similar to that found in ovalbumin, whereas the others are free, as in simple tyrosine peptides.

On the other hand, the tyrosine groups of insulin (Crammer and Neuberger, '43) lysozyme (Fromageot and Schnek, '50), and serum albumin (Tanford and Roberts, '52) appear to be free to ionize without any irreversible change in the protein. Tanford and Roberts have suggested that, because of the abnormally high heats of ionization of the tyrosine hydroxyl groups in serum albumin, they are involved in some kind of loose hydrogen bonding that is easily broken on increasing the *p*H and readily reestablished on decreasing it again. The situation in all these proteins, however, is certainly quite different from that in ovalbumin or ribonuclease.

Breakage of the hydrogen bonds of the tyrosine . . . glutamate type illustrated in figure 4 would permit increased freedom of motion of the side chains after breakage. The entropy effects thus involved would be quite different from those involved in the breaking of the bond in salicylic acid. The possible types of hydrogen bonds between side chains in proteins have been discussed by Laskowski and Scheraga ('54), who have calculated in great detail the possible effects of the formation and breakage of such bonds on the titration curves of proteins. The experimental evidence, however, is still relatively meager.

HELICAL CONFIGURATIONS OF PEPTIDE CHAINS

Peptide chains, like other long chains of atoms, can assume many configurations in space. However, the peptide C═O and N—H groups are highly polar, and capable of forming strong hydrogen bonds. This sets off these chains in sharp contrast to many synthetic high polymers, which are composed of nonpolar residues. A molecule of polyethylene, dissolved in a nonpolar solvent, is an extreme example. The polyethylene chain is free to coil and twist in random fashion into a great number of possible configurations, all of which have nearly the same energy. The statistical distribution of configurations in such a system is determined primarily by entropy considerations (Flory, '53). The peptide chain represents a profoundly different type of system; a very large number of configurations is theoretically possible if the chain is long, but most of them — at least in water and other less polar solvents — are of such relatively high energy that their existence is very improbable, and only very few stable arrangements are probable enough to deserve serious consideration. Some of the major requirements for the existence of such stable configurations of peptide chains have been laid down by Pauling *et al.* ('51; see also Pauling, '53). Briefly, they may be expressed as follows:

1. The interatomic distances and bond angles found in simple peptides should be found, with only slight modification, in peptide chains of any length.
2. Each C═O group should be linked through a hydrogen bond to an N—H, and each N—H to a C═O.

3. The N—H . . . O angle in such hydrogen bonds should be not very far from 180°; a deviation of 30° is considered tolerable. The N to O distance, if such a bond is to be strong, is expected to lie in the range 2.79 ± 0.12 A (Corey and Pauling, '53).

4. The atoms of the amide group, and the two adjoining α-carbon atoms, C_1 and C_2 in figure 6, should be coplanar, or nearly so, because of the large amount of double bond character in the C—N bond denoted by $C{\cdots}N$ in the formula. The C—N distance in this bond is approximately 1.32 A, as compared with 1.47 A for a normal C—N single bond, such as the N—C_2 bond (fig. 6).

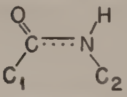


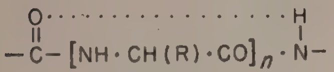
Figure 6

5. The positions of the successive amino acid residues should be equivalent; a combined translation and rotation should therefore carry residue n into the position of residue $n + 1$, a repetition of the same operation should carry it to the position of residue $n + 2$, and so on indefinitely. In this statement, the side chains (R_1, R_2, etc.) attached to the α-carbon atoms are ignored, or they are assumed to be all equivalent.

6. Certain orientations of atoms around valence bonds adjoining α-carbon atoms will be favored over others, for the same reasons that lead to restricted rotation around single bonds in simple organic compounds (Pauling and Corey, '51, '53).

It may be said that the second condition will not necessarily be expected to hold if the peptide chain is immersed in a very strongly hydrogen-bonding medium, such as a concentrated urea solution; the peptide C═O and N—H groups may then form bonds with the solvent molecules, rather than with each other. Moreover, proline and hydroxyproline residues contain no N—H groups, and their nitrogen atoms cannot act as hydrogen bond donors. Also (see fifth condition) if the side chains are markedly different in successive residues, the residues may not be in equivalent positions. Nonequivalent positions may also be imposed by distorting forces, such as those produced by the formation of a disulfide link between two segments of the same chain. Such complications, however, were quite properly ignored in the first formulation of the fundamental conceptions.

These conditions may be met either by structures involving hydrogen bonds between different peptide chains, or by structures with intrachain bonds only. In the latter category the type of structure that most readily meets the conditions stated is a helix. A variety of helices can be constructed which meet these conditions to a close approximation. The most general formulation of these possible helices yet given is that of Low and Grenville-Wells ('53). They fall naturally into two series. One, denoted as the α series, may be represented by the chemical sequence shown in figure 7 with $3n + 4$ atoms in the hydrogen-bonded loop. It is possible to construct models of structures of this series for $n = 2$, 3, 4, or 5 that satisfy the six conditions laid down in figure 7 to a good approximation. The most important is the α-helix of Pauling and Corey, for which $n = 3$, there being 13 atoms in the hydrogen-bonded loop. The structure is shown in figure 8. As this figure indicates, such a helix may be coiled in either a left-handed or a right-handed spiral; the significance of this is considered later. In either case, the interval per residue along the helical axis is 1.47–1.50 A, and there are 3.6–3.7 residues for a complete turn of the helix; the pitch of the helix is therefore close to $3.6 \times 1.5 = 5.4$ A. The C=O group of each residue is hydrogen bonded to the N—H group of the third residue beyond. Another helix of the α series, denoted as the π-helix, is shown in figure 9. Here $n = 4$ and there are 16 atoms in the hydrogen-bonded loop. The C=O group of each residue is hydrogen bonded to the N—H group of the fourth residue beyond. The interval per residue along the helical axis is near 1.14 A, and there are 4.4 residues per turn. The details of the drawing in figure 9 show many of the general characteristics of all these helical structures.



$-\overset{\overset{\mathrm{O}\cdots\cdots}{\|}}{\mathrm{C}}-[\mathrm{NH\cdot CH(R)\cdot CO}]_n\cdot\overset{\overset{\cdots\cdots\mathrm{H}}{|}}{\mathrm{N}}-$

Figure 7

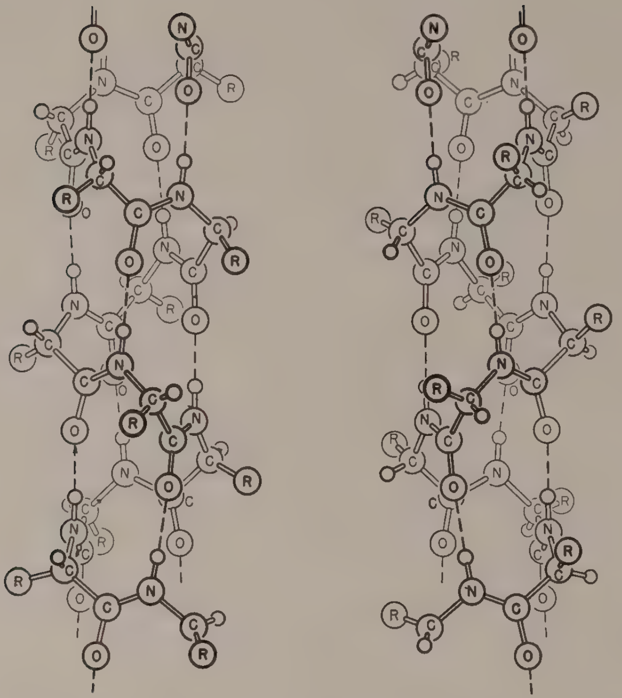
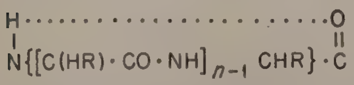


Fig. 8 Perspective drawing of a segment of a left-handed α-helix and of a right-handed α-helix. Note that the two are mirror images if the R groups are removed from the α-carbon atoms and replaced by hydrogen atoms. However, when the R groups are in place, in the L-configuration as shown, then the two helices are no longer mirror images. Unpublished drawing provided by the courtesy of Drs. R. B. Corey and L. Pauling.

Another family of helical configurations has been denoted as the γ series by Low and Grenville-Wells. This corresponds to the sequence shown in figure 10 with $3(n-1)+5$ atoms in the hydrogen-bonded loop. For the γ-helix of Pauling and Corey ('51), $n = 5$. The helices of this family are quite distinct from those of the α series; a helix of one series cannot be converted into one of the other without unfolding and rewinding of the peptide chain, nor can a region of one succeed a region of the other along the same axis. Since as yet there is no



$$\begin{array}{l}\mathrm{H}\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\mathrm{O}\\ | \qquad\qquad\qquad\qquad\qquad\quad \| \\ \mathrm{N}\{[\mathrm{C(HR)\cdot CO\cdot NH}]_{n-1}\,\mathrm{CHR}\}\cdot\mathrm{C}\end{array}$$

Figure 10

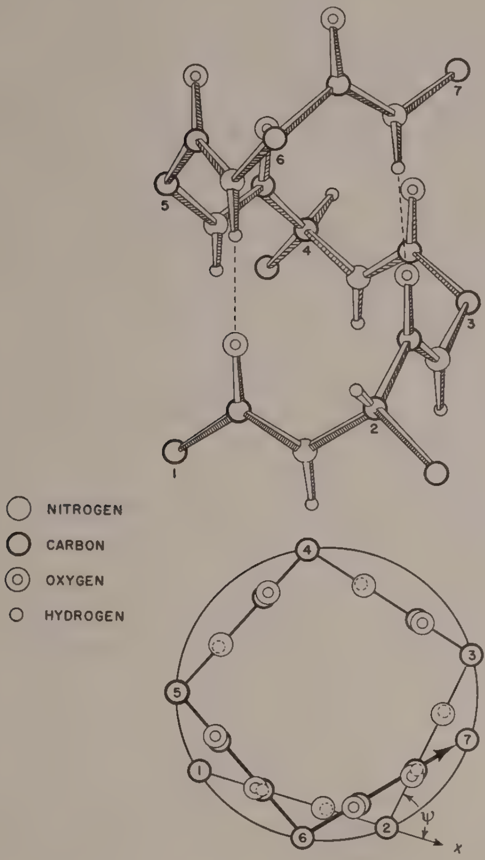


Fig. 9 The upper portion is a perspective drawing of a right-handed π-helix (Low and Grenville-Wells, '53). The successive α-carbon atoms in the peptide chain are numbered consecutively. The β-carbon and hydrogen-atom positions for L-amino acids are shown attached to $C_2(\alpha)$ and $C_4(\alpha)$. Note that C_1, C_2, and the atoms of the intervening –CO·NH– group are all coplanar; the same is true generally for C_n, C_{n+1}, and the intervening –CO·NH– group atoms.

In the lower diagram, the planes of these successive residues are seen in projection from the point of view of an observer looking down along the helical axis. The angle ψ represents the horizontal component of the rotation between the planes of successive amino acid residues. The vertical rise, along the helical axis, is approximately 1.14 A, from any atom in the n'th chain residue to the corresponding atom in the $(n+1)$'th residue.

evidence to indicate that helices of the γ series have been observed in nature, they will not be considered further here.

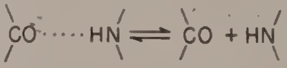
Extended configurations of peptide chains, in which the —CO . . . HN— hydrogen bonds cross-link residues of different chains, are found in such structures as silk fibroin and β-keratin. The most promising models yet developed for the description of such structures are the parallel and antiparallel "pleated sheet" structures of Pauling and Corey ('51, '53). Strong evidence has been produced (Marsh *et al.*, '55) that silk fibroin is an antiparallel pleated sheet structure. However, the principal concern here is with the globular proteins, and no good evidence for the presence of such extended peptide chain configurations in the internal structure of globular proteins has yet been produced. Hence, these structures also will not be considered further here.

For certain synthetic polypeptides, such as poly-γ-methyl-ʟ-glutamate and ε-carbobenzoxy-poly-ʟ-lysine, X-ray diffraction studies (Cochran and Crick, '52; Yakel, '53) leave little doubt of the reality of the α-helix configuration. The fit between the calculated and the observed diffraction patterns is remarkable. The evidence for the presence of α-helices in proteins is less direct, and has been the subject of much debate. However, the total weight of the evidence — which is discussed in more detail by Low and Edsall ('56) — does appear to be distinctly in favor of the existence of the α-helix as an important element of structure, both in fibrous proteins of the keratin-myosin-epidermin-fibrinogen class and in many globular proteins. For instance, Arndt and Riley ('55) have made extensive studies of the X-ray scattering from a large number of globular proteins, studied as amorphous powders. The observed distribution of scattered intensity was compared with the intensity distributions calculated from the parameters of a number of possible well-defined chain configurations.

Two possible configurations of the α, π, and other helices must here be considered. The α-helix shown on the right in figure 8 is a right-handed helix; the α—C—β—C bond has a vertical component in the same direction as the vertical component of the N—H bond. In the left-handed helix, the two are opposed. A left-handed helix of L-amino acid residues is the mirror image of a right-handed helix of D-amino acid residues; but if the configuration of the residues is L in both cases, then the left- and right-handed helices are not mirror images, as is readily apparent from figure 8. The studies of Arndt and Riley showed, for more than 20 globular proteins, that the observed X-ray scattering distribution was in better accord with the scattering of a left-handed α-helix (denoted by them as α_1) than with any of the other models considered. They pointed out, however, that the existence of alternative configurations over limited sections of the peptide chains was compatible with their data. In particular, the α_2-helix (right-handed) could occur as an important fraction of the molecule, although it was considered unlikely to be the major constituent. The π_1- and π_2-helices were also not excluded as possible constituents of some of the peptide chains.

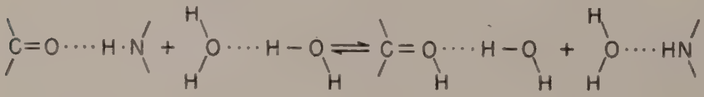
STABILITY OF HELICAL CONFIGURATIONS

Helical configurations of peptide chains may be stable or unstable, as compared with the more randomly coiled configurations that are possible when the —C=O . . . H—N— hydrogen bonds are broken and the regular helix structure is unfolded. The free energy of unfolding (ΔF_{unf}) is of course the determining factor; if this is negative, the helix (if it is formed at all) will be unstable. A very important critical examination of the whole problem has been given by Schellman ('55b), which is closely related to the earlier discussion by Kauzmann ('54). Heat is absorbed when a-CO . . . HN— bond is broken; for each bond, this contributes a positive term ΔH_{res} to the free energy of unfolding. On the other hand, there is a gain in entropy caused by unfolding, since the unfolded chain is free to assume many more configurations than is the well-ordered helix. There are some terminal —C=O or —N—H groups at each end of the helix — four of each for an α-helix, if the terminal amino and carboxyl groups are also counted — which, because they are terminal, cannot enter into intrachain CO . . . HN linkages; this circumstance must be allowed for in calculating ΔF_{unf}. The general conclusion is that ΔF_{unf} is negative for short chains, positive for long ones; that is, the helical configuration is stable if the chain is above a certain critical length, whereas a more nearly random configuration is expected for chains below this length. The effect of the surrounding medium on the value of ΔH_{res} is of the first importance in these calculations. In a medium of low dielectric constant, incapable of hydrogen bond formation with the groups in the peptide chain, the reaction involved may be represented as in equation (1).



$$\mathrm{CO\cdots HN \rightleftharpoons CO + HN} \qquad (1)$$

and ΔH_{res} is of the order of 8 kcal/mole (Pauling *et al.*, '51). In water, on the other hand, the reaction may be written, following Kauzmann ('54):



$$\mathrm{C{=}O\cdots H{\cdot}N + H_2O\cdots H{-}OH \rightleftharpoons C{=}O\cdots H{-}OH + H_2O\cdots HN} \qquad (2)$$

The net value of ΔH_{res} for this reaction, which may be considered as hydrogen bond interchange rather than hydrogen bond breakage, is naturally much less than for the other. Schellman ('55a) estimates a value of 1.5 kcal/mole, from data on the association of urea molecules in aqueous solution and the temperature coefficient of the association constant. Thus the critical chain length for helix formation should be much longer in aqueous solution than for a polypeptide in the dry state, or in a solvent which does not form hydrogen bonds.

One significant factor affecting the stability of a helix in aqueous solution is not dealt with in Schellman's calculations, as Professor Paul Doty has pointed out in personal discussions. The hydrogen bonding of water molecules to the —C=O and —N—H groups, which occurs [see equation (2)] when a helix is unfolded, contributes a negative term to the entropy of unfolding, owing to the orientation of the attached water molecules, and the resulting restriction on their freedom of rotation. The magnitude of this effect is not established, but it serves to counteract to some extent the gain in entropy of the polypeptide chain itself, which results from the unfolding of the helix. Qualitatively, the inclusion of this factor leaves Schellman's conclusions unaltered; but allowance for this added entropy effect would lead to the expectation that the critical chain length for the formation of a stable helix would be somewhat less than that expected from Schellman's calculations.

The stability of actual polypeptide chains, however, is much affected by cross links between the chains, and by interactions between the side chains of the amino acid residues. The presence of disulfide or other cross links between different chains decreases the number of possible configurations that the unfolded chains can assume, and thus tends to stabilize the helical arrangement. On the other hand, it may be remarked here that if a single peptide chain is bent into loops by intrachain disulfide bonds, limits are imposed on the lengths of the segments that can proceed in any one direction before they undergo the bending required by the existence of the loop (fig. 11). This factor, therefore, would tend to diminish the kind of regularity required for the formation of an α-helix, and favors more irregular configurations.

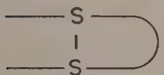


Figure 11

Side-chain interactions may be of great importance for the stability of helices; a series of adjacent side chains with the same sign of charge gives rise to repulsive interactions, and hence favors extended configurations. If the adjacent charges are of opposite sign, or if hydrogen bond formation occurs between adjacent side chains, compact configurations such as the α-helix are favored.

Relatively little experimental evidence is yet available for the direct testing of Schellman's conclusions. In general it is true that long-chain peptides are most likely to be found in the compact α configuration, as judged by X-ray and infrared studies. Unfolded chains often aggregate, at least in the solid state, to form fibers aligned in the β configuration. Doty *et al.* ('54) have prepared poly-γ-benzyl-L-glutamate of widely varying degrees of polymerization, each preparation being of highly uniform chain length. In weakly interacting solvents such as chloroform, these exist in the α form if the molecular weight is high; strongly hydrogen-bonding solvents, such as dichloroacetic acid, lead to unfolding of the polymer into a randomly coiled configuration, regardless of molecular weight. Very low molecular weight polymers, in weakly interacting solvents, exist partly as aggregates in the extended β form, which dissociate on dilution to give a solvated form. All this is qualitatively in accord with the theoretical discussion outlined, but the most interesting field of study, that of the configuration of large water-soluble polypeptides in aqueous solution, is just beginning.

TURNING OF PEPTIDE CHAINS AROUND CORNERS

The globular proteins are in general so compact that the constituent peptide chains must make turns at intervals and reverse direction. Thus ribonuclease, with 126 residues, would be nearly 190 A long if it were a straight α-helix. The largest axis in the unit cell of the crystal, however, is only a little over 50 A (Carlisle and Scouloudi, '51), and sedimentation and diffusion measurements on the molecule in solution (Anfinsen *et al.*, '54) give a frictional ratio near 1.3, typical of many globular proteins and indicating no marked asymmetry. Moreover the chemical evidence indicates that ribonuclease, although it consists of only a single peptide chain, contains four disulfide groups (Anfinsen *et al.*, '54); hence turning of corners between segments of the peptide chain must occur to permit the formation of loops fastened by the disulfide cross links. Exactly similar arguments hold in serum albumin, which, if a molecular weight near 66,000 is assumed, contains some 560 residues and 15 disulfide cross links, but apparently consists of only a single peptide chain (van Vunakis and Brand, '51; Thompson, '54; Hunter and McDuffie, '56). An extended α-helix of 560 residues would be 840 A in length, but the longest dimension in the unit cell of air-dried human serum mercaptalbumin mercury dimer crystals is only 148 A (Low, '52b); and the behavior of the molecule in solution is compatible only with the assumption that the axial ratio of the molecule in solution is less than 4:1 — probably significantly less (Edsall, '53b, p. 721). The very careful studies of low-angle X-ray scattering in albumin solutions (Anderegg, *et al.*, '55) indeed tend to favor an even more compact model, possibly less than 100 A in its longest dimension, although the results are not conclusive. Here again, repeated folding of the peptide chain is in any case demanded by the presence of the disulfide linkages. In the very different structure of horse hemoglobin, which apparently contains no disulfide cross links (Ingram, '55), the number of crystallographic chains in the molecule deduced from the work of Bragg and Perutz is seventeen, but there are only six peptide chains. Actually, the number of discontinuities may be even larger (see the discussion of Low and Edsall, '56).

Such structures clearly require that peptide chains must turn corners and reverse direction. It has generally been suspected that prolyl residues are involved in such turns, because of the geometry of the closed pyrrolidine ring and the lack of an —NH group for hydrogen bonding. If the principal straight portions of the peptide chains are assumed to be in the form of α-helices, the effect of the introduction of an L-prolyl residue is quite different for a right-handed and for a left-handed helix, as has been described elsewhere (see Low and Edsall, '56). In the right-handed helix, a reasonably planar proline ring can be constructed that involves the elimination of one hydrogen bond and the lengthening of another, but that could be inserted without completely disrupting the helical configuration. In a left-handed helix, however, the prolyl residue would quite disrupt the continuity of the helix; achievement of complete reversal of direction of the helix would involve alteration in the arrangement of several amino acid residues, one or two on each side of the proline. Several detailed mechanisms whereby this might occur have been discussed (Edsall, '54a).

The number of proline residues in certain proteins — 4 in ribonuclease, and approximately 30 in human and bovine serum albumin — appear quite adequate to account for the number of turns in the chain required by the disulfide linkages, and by the general size and shape of the molecule. Evidence for the locations of the proline residues in the chain sequence is not yet available; and in some proteins there may be more turns than proline residues. Certainly, other possible modes of turning deserve to be considered, and an alternative, more direct stereochemical mechanism for achieving a 180° turn in the axis of a left-handed helix has been proposed by Lindley ('55). It depends on the existence of certain residue sequences and the formation of hydrogen bonds between certain side chains, such as glutamyl residues, and the main peptide chain.

Recent evidence on the amino acid residue sequence in ribonuclease (C. H. W. Hirs, W. H. Stein, and S. Moore, personal communication) has shown that two of the four prolines are in a single segment, 15 residues long, at the C-terminal end of the chain. Some at least of the loops, imposed by the presence of four disulfide linkages in the single chain of the ribonuclease molecule, must therefore involve reversal of the chain direction by means of residue sequences not involving proline. Indeed, the possibility should be reckoned with that proline is not involved at all in such turns. Lindley ('55) has suggested that proline residues may serve to provide a transition region between a left-handed and a right-handed region of an α-helix, and this may conceivably be their chief stereochemical function in protein structure.

STEREOCHEMISTRY OF INSULIN

The diagrammatic representation of the insulin structure shown in figure 1 is obviously not a stereochemical model. Many possibilities for the actual spatial arrangement are still left open. X-ray studies of Low ('52a) on air-dried crystals of acid insulin sulfate offer important evidence that should be considered in the formulation of any model. The distribution of peaks in the vector structure (Patterson series) indicates the presence of cylindrical rod-like elements, stacked in closely packed array, and parallel to the a axis of the unit cell. The a axis in the air-dried crystals is 44 A long. This is very close to the length that would be expected for the B chain of insulin, with 30 residues, if it were coiled in an α-helix, the length along the helical axis being 1.5 A per residue. This correspondence suggests that the α-helix may be a suitable model for the B chain. Supporting evidence comes from the study by Arndt and Riley ('55), which has already been discussed, and which indicated the α_1-helix (left-handed) as the predominant configuration. However, even if the B chain is an α-helix, the section of the A chain between residues 6 and 11, which is closed by the intrachain disulfide link (fig. 2) cannot assume any simple helical configuration, as is readily seen if one attempts to construct a model. On the other hand, the two terminal segments of the A chain may assume an α-helix configuration. Lindley and Rollett ('55) have shown that the region of the chain between A_6 and A_{11} may be twisted into a more regular configuration if it is assumed to join two segments of a chain that have different screw directions. They take the residues from A_1 to A_9 as forming a left-handed coil, and those from A_9 to A_{21} as forming a right-handed coil. Then the A_6–A_{11} region forms a sort of figure 8, as required by the change of hand in the coil. They have developed a very detailed model, in which only two of the hydrogen bonds in the A chain are broken. In this model, the helical axis of the A_9–A_{21} segment is parallel to a right-handed B chain α-helix. The helical axis of the A_1–A_7 segment is in the same plane as the axes of the B chain and as the A_9–A_{21} axis, but is tilted away from B at an angle of 30°.

Independently, Low has constructed a somewhat different insulin model, which is discussed by Low and Edsall, '56. This also involves a change of hand, from a left-handed A_1–A_8 segment to a right-handed A_{11}–A_{21} segment. In this model, however, the B chain is a left-handed, not a right-handed helix.

The two parts of the A chain in this model do not lie in the same plane, but there is a lateral shift between them. Thus this model appears in an ''end-on'' projection as an area of three closely packed helices. The helices in this model are predominantly left-handed, whereas in the Lindley and Rollett model they are predominantly right-handed. Both structures are plausible; neither is proved. They are exploratory devices that should serve as useful guides in further studies. Such studies must explain, among many other matters, the remarkable tendency of insulin molecules to polymerize reversibly in aqueous solution. The smallest unit readily obtainable under ordinary conditions in water is of molecular weight near 12,000, with two A and two B chains; and in neutral solution the prevalent forms are three or four times as large as this. However, in guanidine hydrochloride solution, isoelectric insulin dissociates, apparently reversibly, into units of 6000 molecular weight, as judged by osmotic pressure measurements (Kupke and Linderstrøm-Lang, '54).

RIBONUCLEASE

This relatively simple protein has repeatedly been referred to in the preceding discussion. It is certainly a highly compact structure; its single peptide chain with approximately 126 residues (Hirs *et al.,* '54) is evidently looped back upon itself into at least four or five shorter segments. The four disulfide linkages impose some of these loops, and the presence of three hydrogen bonds involving tyrosine residues (Shugar, '52; Tanford *et al.,* '55) of the sort already discussed must serve as an additional stabilizing factor in maintaining the molecule in its compactly folded configuration. Anfinsen *et al.* ('54) have presented a tentative picture of the molecular pattern, but their own further work and that of Hirs, Stein, and Moore has already made this picture far out of date. R. R. Redfield (personal communication) reports that the N-terminal sequence is now established for the first seven residues as Lys.Glu.Thr.Ala.Ala.Ala.Lys. Several partial internal sequences are also known. The C-terminal residue is Val, and the latest findings indicate Ala and Ser as the two adjoining residues, probably in the order Ala.Ser.Val.

A recent study from the Carlsberg Laboratory (Anfinsen *et al.*, '55) shows that the enzymic activity of ribonuclease, as judged by its attack on ribonucleic acid, is essentially unimpaired by dissolving it in 8 *M* urea, although its increased intrinsic viscosity shows that the enzyme has undergone a considerable degree of unfolding. Also, partial digestion of ribonuclease by the enzyme subtilisin (from *Bacillus subtilis*) leads to a modified but fully active form in which a new N-terminal group can be demonstrated. Even restricted pepsin digestion, however, destroys activity. Clearly, enzyme activity does not require the intact configuration of the native molecule, although it cannot yet be said just what the essential features for enzyme activity may be.

SERUM ALBUMIN

Bovine and human serum albumin are strikingly alike in size and in their general physical properties. In amino acid composition also they are closely similar, although the work of Brand ('46) has shown that they differ quite distinctly in their content of methionine, serine, threonine, tyrosine, glycine, valine, leucine, isoleucine, phenylalanine, and probably tryptophan. There may be minor differences in other amino acids also, but the number of positively and negatively charged groups — almost 100 of each per molecule of isoelectric protein — is almost identical for both. The molecular weight of both has generally been taken as 69,000, largely on the basis of the very careful osmotic pressure measurements of Scatchard *et al.* ('46) on bovine serum albumin. The X-ray studies of Low ('52b) on human albumin, however, give a value of 65,000 from the dimensions of the unit cell of the crystal and the composition and density. Some other recent studies (summarized by Anderegg *et al.*, '55) give a nearly identical value for bovine albumin. Tentatively, the molecular weight is taken as 66,000; this gives, from Brand's data, fifteen disulfide linkages per molecule in both human and bovine albumin. The major constituent of both preparations is mercaptalbumin (ASH; see Hughes, '50) with one reactive sulfhydryl group per molecule of albumin. This is readily separated from the rest of the albumin fraction by crystallization as the mercury derivative ASHgSA. The most careful determinations of N-terminal groups (van Vunakis and Brand, '51; Thompson, '54) have revealed only one per molecule of albumin. This is aspartyl in both proteins, although the next adjoining residue is alanyl in human, threonyl in bovine serum albumin (Thompson, '54). Studies with carboxypeptidase (White *et al.,* '55) give results that are not so conclusive in this respect. Human serum albumin appears to have one C-terminal leucine, with alanine, valine, and glycine also being released more slowly, in that order. Bovine serum albumin released one mole of C-terminal alanine very rapidly, with a second mole of alanine plus leucine, threonine, valine, and serine following at slower but appreciable rates. On the whole, the evidence accords with the view that both the serum albumins consist of a single peptide chain, of some 560 residues, cross linked in fifteen places by disulfide links. This conclusion has been reinforced, for human albumin, by the work of Hunter and McDuffie ('56) at Harvard. They have reduced all the disulfide bonds of human serum albumin by the action of thioglycolate in mildly alkaline solution, and then alkylated the resulting $—SH$ groups to $—S\cdot CH_2\cdot CO\cdot NH_2$ groups by treatment with iodoacetamide. The resulting reduced and alkylated protein was itself insoluble in water but was soluble as a complex with dodecyl sulfate. The complex contained approximately 95 moles of dodecyl sulfate per mole of albumin, and molecular weight determinations by light scattering, and sedimentation and viscosity measurements, gave a value of 88,000–93,000, approximately what was to be expected for such a complex if the albumin molecule had not been broken down into smaller subunits by the disulfide splitting. They conclude that "the human serum albumin molecule is a continuous peptide chain internally cross linked by disulfide bonds." The number of proline residues (approximately 29 for human, 32 for bovine albumin) is ample to provide the necessary turning points for reversal of chain direction in the loops produced by the disulfide cross links. It should be emphasized again, however, that proline is not necessarily involved in these turning points, and other possibilities such as those proposed by Lindley ('55) must be considered. Thus the general pattern of the albumin molecule appears to be not unlike that of ribonuclease already indicated, but on a much larger scale, with a peptide chain more than four times as long as that of ribonuclease, and four times as many disulfide cross links.

One careful piece of work suggests that bovine albumin, at least, may consist of more than one peptide chain. Reichmann and Colvin ('56) have oxidized albumin with performic acid, which oxidizes each disulfide bond to a pair of sulfonate groups, and have found the molecular weight, as determined by osmotic pressure, to decrease to 19,000; or, as determined by light scattering and sedimentation equilibrium, to 30,000. If only disulfide bonds are broken by performic acid, these results would imply that bovine albumin consists of three or four peptide chains, bound together by disulfide bonds. It is possible, however, that other reactions, involving the breakage of peptide chains, may be produced by this reagent. Final judgment as to whether albumin consists of one or of several peptide chains must therefore be postponed.

Although the portions of the serum albumin molecule are tied together by the fifteen disulfide bridges, the structure appears to possess considerable flexibility (Scatchard, '52). The large and reversible increase in optical rotation and intrinsic viscosity that occurs in acid solutions of albumin at *p*H 2–4 (Yang and Foster, '54) appears to be best explained by a reversible swelling or unfolding. The electrostatic effects on proton binding in acid solution (Tanford, '55) when interpreted in terms of a model of the protein that is assumed to be a uniformly charged sphere, lead to the result that the radius of this sphere must increase from about 35 A at *p*H 5 to about 65 A at *p*H 2.5; whereas Tanford points out, by contrast, that the results of similar titrations on β-lactoglobulin by R. K. Cannan and his coworkers give no change over this *p*H range in the radius of the equivalent sphere. These charged sphere models should not be taken too seriously, but qualitatively at least, the titration data do point to reversible swelling of albumin in acid, presumably owing to the repulsions arising when the net charge on the molecule is increased. Such a phenomenon would be closely related to the swelling of ionic gel networks (Flory, '53, pp. 584–589). Something similar may occur in alkaline solutions, as judged by the large heat of ionization of the tyrosine hydroxyl groups, and other indications of breaking of hydrogen bonds (Tanford and Roberts, '52). In any case, the alterations occurring appear to be largely, although not completely, reversible when the molecule is brought back to the isoelectric condition. There is other evidence concerning the flexibility of serum albumin, which will not be discussed here. The revealing studies of Kauzmann and his associates, on the urea denaturation of serum albumin and ovalbumin, are of outstanding importance for our picture of the structure of these proteins, and are discussed elsewhere in this symposium.

The general shape of the isoelectric, or nearly isoelectric, serum albumin molecule is not very far from spherical. The difficulties of evaluating this shape with any degree of exactness were discussed 3 years ago (Edsall, '53a); proposed models have ranged from a prolate ellipsoid, 150 A by 38 A, through prismatic models of somewhat similar dimensions, to an oblate ellipsoid with an axial ratio of 1:3.5. The low-angle X-ray scattering work of Anderegg *et al.* ('55) — probably the most careful work of this type on a protein that has yet been done — is very difficult to reconcile with a prolate model. They suggest, very tentatively, several possible models, all of which are less than 106 A long in their greatest dimension, and point out how either one of two of these could be packed into the crystal unit cell determined by Low ('52b). An extension of their study to acid solutions might throw light on the problem of the swelling of the albumin molecule in such solutions; for X-ray scattering should measure the radius of gyration of the protein molecule itself, as it exists in solution, including any internal water of hydration, but not water of hydration on the surface.

The discovery by Hughes ('50) of the single sulfhydryl group in mercaptalbumin, and the remarkable reaction by which mercury binds two mercaptalbumin molecules together, has led to further study of the quantitative aspects of the reaction, light scattering being used to follow the process (Edelhoch *et al.*, '53; Edsall *et al.*, '54; Edsall, '54b). The reaction is a well-defined and reversible process, both for human and for bovine mercaptalbumin, following equation (3).

$$\mathrm{ASHgX} + \mathrm{ASH} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AS-Hg-SA} + \mathrm{H^+} + \mathrm{X^-} \qquad (3)$$

(Here X may be chloride or acetate, possibly sometimes hydroxyl.) The standard enthalpy of activation is near 20 kcal/mole; the standard entropy of activation is positive, whereas most processes, involving the union of two molecules to form one, have a large negative entropy of activation. (Also the formation of the H^+ and X^- ions in the reaction as written would be expected to involve a negative contribution to the standard entropy.) Dimerization with an organic bifunctional mercurial, XHgRHgX — where R is a dioxane ring with two methylene groups in the 1 and 4 positions, to which the mercury atoms are attached — to form ASHgRHgSA proceeds some 2000 times as rapidly as the reaction shown. This is presumably due to the decrease in steric resistance to the approach of the two albumin molecules when they are more widely separated by the large organic residue between the two mercury atoms. The two sulfur atoms of the two albumin —SH groups are separated by about 5 A in ASHgSA, by about 15 A in ASHg·R·HgSA (Edsall, '54b), and this difference should allow for more free play for the two components of the dimer relative to each other in the latter case. The enthalpy of activation for the formation of ASHgRHgSA is again near 20 kcal/mole, and the standard entropy of activation is even more positive than with the simple mercury dimer, approximately 14 cal deg^{-1} $mole^{-1}$ at pH 5.5 and 26 cal deg^{-1} $mole^{-1}$ at pH 6. This positive entropy term may indicate an unfolding of the albumin molecule as a prelude to the dimerization reaction; it may have to do with a dehydration process associated with the neutralization of charged groups, when the two monomers approach to form the dimer; or it may be caused by other factors, as yet unrecognized. Bovine and human mercaptalbumin behave similarly in these reactions, but the association constant for the dimerization equilibrium is markedly higher for the bovine than for the human protein, according to the work of R. H. Maybury and of Cyril M. Kay in this laboratory. The bovine material also appears to have greater uniformity of behavior; the dimerization process follows the theoretical relations for a second-order reaction with astonishingly high precision, over a wide range of values of pH, ionic strength, and temperature, although the absolute value of the velocity constant k_2 may differ by several hundredfold according to conditions.

Note added in proof. Mr. Kay has now shown that the dimerization reaction of serum albumin proceeds about four times as rapidly at 25°C. in 8 M urea as in water, but with a considerably lower energy of activation.

DISCUSSION

HARKER[1]: The Patterson function of insulin does indeed contain rod-like regions of high density, but this does not necessarily indicate that the structure of insulin itself consists of rods. It is fairly easy to set up hypothetical structures that have no rod-like character, but of which the Patterson functions consist very definitely of rods. On the other hand, a structure of parallel rods has a Patterson function also consisting of rods.

Frequently, a Patterson function consists of a three-dimensional array of peaks distributed with great regularity; in

[1] David Harker, Polytechnic Institute of Brooklyn.

such cases, it is possible to select rows of peaks in any of a number of directions and then to assume that they are the Patterson images of rather knotty rods in the structure itself. This is a dangerous procedure, but it has been used by the Cambridge group of X-ray crystallographers in discussing hemoglobin and myoglobin.

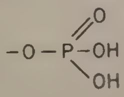
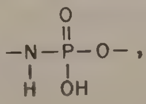
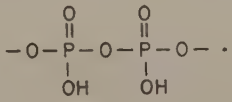
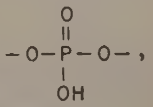
Our own results on ribonuclease crystals are not compatible with parallel rod-like structures in the molecules. There might be rods in several directions, but this seems unlikely. The same situation seems to exist for lysozyme crystals. My feeling is that rods are almost certainly absent from the ribonuclease and lysozyme structures, probably absent from hemoblobin and myoglobin, and not proved present in insulin.

My colleague, Dr. M. V. King, has prepared crystals of ribonuclease with and without the presence of dye molecules containing uranium atoms. The X-ray diffraction data from these two kinds of crystals can be used to find the electron density distribution in the undyed crystal. We have done this for one projection; no evidence of parallel rod-like structures appeared. The same seems to be true of a projection of the electron density of hemoglobin prepared by Dr. Perutz of Cambridge.

We crystallographers are all very anxious to find out what the structure of a crystalline protein really is — no doubt we shall before too long. In the meantime it seems unlikely that the globular proteins have structures consisting of parallel rods.

Edsall: I certainly would not insist that rod-like or cylindrical patterns observed in a Patterson diagram of a protein crystal must mean polypeptide chains in the actual structure. There must certainly be other possible interpretations of such observations, and Dr. Harker is far more competent than I to suggest what they might be. However, I am impressed by the fact that these rods, observed by Dr. Low in three-dimensional Pattersons of the crystals of acid insulin sulfate, are parallel to the *a* axis, and that the length of this axis (44 A) is very close to the length expected for the longer (B) chain of insulin if it is indeed an α-helix. Moreover, I do take seriously the suggestive evidence from the X-ray scattering work of Arndt and Riley, which they interpret as favoring the α-helix structure for the major part of the insulin molecule. I do not yet think any of this evidence is final, but I would hold to the opinion that the weight of the evidence for insulin is in line with this view.

Perlmann[2]: Dr. Haurowitz and Dr. Edsall have discussed the role of the disulfide linkages in proteins and their participation as inter- and intramolecular bonds. During the past 4 years, we have been able to show that the phosphorus in phosphoproteins is present in the form of monoesters as well as in structural units such as those of diesters and pyrophosphates, thus adding a new type of cross link to that of the disulfide bonds. As in the sulfur bridges, the phosphorus in proteins can either link peptide chains or close a ring.

A demonstration of these linkages was achieved by enzymic hydrolysis with the use of enzymes specific for diester and pyrophosphate bonds. This brought about their transformation into monoesters, which could then be released in the form of inorganic phosphate with the aid of a variety of phosphomonoesterases from plants and from mammalian tissue. Such an experimental approach not only determines the nature of these linkages but also the proportions in which they are present in a protein. Thus α-casein with a phosphorus content of 1% contains 40% of its phosphorus as monoesters,  $-\mathrm{O}-\mathrm{P}\begin{matrix}/\!\!/\mathrm{O}\\ -\mathrm{OH}\\ \diagdown\mathrm{OH}\end{matrix}$, 40% as diesters,  $-\underset{\mathrm{H}}{\underset{|}{\mathrm{N}}}-\underset{\mathrm{OH}}{\underset{|}{\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}}}-\mathrm{O}-$, and 20% as pyrophosphate,  $-\mathrm{O}-\underset{\mathrm{OH}}{\underset{|}{\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}}}-\mathrm{O}-\underset{\mathrm{OH}}{\underset{|}{\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}}}-\mathrm{O}-$. In β-casein, all of the phosphorus is present in the form of diester linkages,  $-\mathrm{O}-\underset{\mathrm{OH}}{\underset{|}{\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}}}-\mathrm{O}-$, between peptide chains. A breakage of the cross linkages leads to splitting of both α- and β-casein into smaller subunits.

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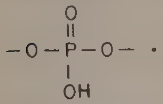
[2] Gertrude E. Perlmann, Rockefeller Institute for Medical Research.

In pepsin, a protein with one phosphorus atom per molecule, the phosphorus is present as a diester of the type,  $-\mathrm{O}-\underset{\underset{\mathrm{OH}}{|}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}-\mathrm{O}-$ · Since it is known from the work of Herriott and of Williamson and Passmann that pepsin is a protein with a single peptide chain, the phosphorus must serve to link two sites of the peptide chain in a cyclic loop.

Kauzmann[3]: You will find evidence for the strength of this bond discussed at length in a paper by Schellman ('55b).

Simpson[4]: The unit molecule of insulin, according to the structure finally proposed by Sanger, has a molecular weight of approximately 6000, and I should like to draw it as he does. However, the structure can also be drawn as a four-chain unit molecule (not a dimer). Such a structure is still in accord with Sanger's results. Since the molecular weight determinations of 6000 were performed under severe conditions, the possibility still exists that the unit molecular weight is 12,000, a molecule of four chains. I wonder how the hypothesis of either the α- or the π-helix fits such an insulin molecule.

Edsall: I think you can fit in the α-helix very well with the system of four chains. In fact, Low ('52) originally built up her model on the supposition that it was a 12,000 and not a 6000 molecular weight molecule. Her packing diagram, which I showed earlier in my talk, was really based on that assumption. Two molecules, each of approximately 6000 molecular weight — the precise value is 5733 for beef insulin — must presumably pack very closely together to form a unit twice as large, which is the usual stable form in acid aqueous solution. The packing unit in the crystal, therefore, is this double unit with two A and two B chains, and a molecular weight near 12,000 (more exactly, 11,466). However, the evidence from the work of Sanger, of Craig, and of Linderstrøm-Lang does point strongly, I think decisively, to the view that the fundamental molecule contains only one A and one B chain.

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[3] Walter Kauzmann, Princeton University.

[4] M. V. Simpson, Yale University.

Kupke and Linderstrøm-Lang ('54) found that osmotic pressure of insulin in guanidine hydrochloride solutions was directly suggestive of the 6000 molecular weight. Harfenist and Craig's studies ('52) were carried out under more drastic conditions. They involved preparing 2,4-dinitrophenyl derivatives of insulin, and then subjecting these to countercurrent distribution, by use of butanol and aqueous dichloroacetic acid. Linderstrøm-Lang's experiments, however, were carried out under much milder conditions, and they lead to the same conclusions.

Ellenbogen[5]: In regard to the possibility of a small insulin molecule (6000), we have fractionated pancreas and did in fact obtain a small-molecular-weight component of that order of magnitude. When tested biologically, however, it was inactive.

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STERIC SPECIFICITY OF HYDROGEN TRANSFER IN PYRIDINE NUCLEOTIDE DEHYDROGENASE REACTIONS[1]

BIRGIT VENNESLAND

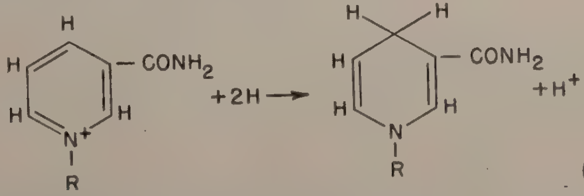
Department of Biochemistry, University of Chicago, Chicago

ONE FIGURE

During the past 5 years a group at the University of Chicago have been using deuterium as a tracer to study hydrogen transfer in reactions catalyzed by pyridine nucleotide dehydrogenases (Westheimer *et al.*, '51; Fisher *et al.*, '53; Loewus *et al.*, '53; Loewus, Westheimer, and Vennesland, '53; Vennesland and Westheimer, '54; Loewus, Tchen, and Vennesland, '55; Talalay *et al.*, '55). This report will be concerned primarily with the results of these investigations. The problem was originally undertaken in collaboration with Prof. F. H. Westheimer, and many individuals have made important contributions to various aspects of the work. It should be understood that this is a report for the group as a whole.

CHEMICAL REDUCTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE

In order to provide a background for a discussion of the more recent experiments, earlier results may be summarized briefly. Equation (1) shows a reaction catalyzed by a pyridine nucleotide dehydrogenase.



(1)

(R represents the ribose-pyrophosphate-ribose-adenine moiety of DPN.)

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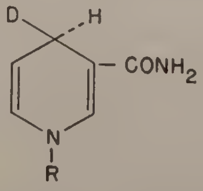
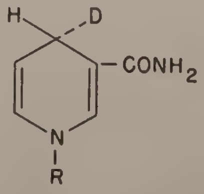
[1] This work has been supported in part by grants from the National Institutes of Health, United States Public Health Service, the American Cancer Society, and from the National Science Foundation.

This equation represents the oxidation of a substrate by diphosphopyridine nucleotide (DPN). The substrate of the dehydrogenase usually loses two hydrogen atoms (or two protons and two electrons). One proton and two electrons are added to the nicotinamide ring and a proton is released into the medium. The hydrogen is added to the nicotinamide ring at position 4. The correct structure of the reduced pyridine nucleotide was first elucidated by Pullman *et al.* ('54) and has been confirmed by Talalay *et al.* ('55) and Loewus, Vennesland, and Harris ('55). This demonstration that the reduction occurs at position 4, and not at position 2 or 6, was an outgrowth of our first experiments with deuterium, which showed that one atom of nonexchangeable deuterium is introduced into DPN on reduction with sodium hydrosulfite in a medium of deuterium oxide. In all the experimental work cited, exchangeable deuterium is washed out with water prior to analysis. The concern throughout is with nonexchangeable deuterium only.

In the chemical reduction of DPN by hydrosulfite, the extra hydrogen in the reduced DPN must be derived from the medium, since the reducing agent ($Na_2S_2O_4$) contains no hydrogen.[2] The introduction of deuterium creates a new asymmetric carbon atom at position 4 of the pyridine ring. Since deuterium may be introduced on either side of the plane of the ring, a mixture of diastereomers is obtained in the chemical reduction. These may be represented by the following formulas.





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[2] The mechanism of this reduction has been studied by Yarmolinsky and Colowick ('54).

THE ENZYMIC REDUCTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE BY ALCOHOLS

Many of the enzymic reactions catalyzed by the pyridine nucleotide dehydrogenases involve the oxidation of primary or secondary alcohols to carbonyl compounds. In four such reactions, it has been shown that the added hydrogen in the reduced DPN, which is formed by enzymic reduction, is derived directly from the substrate molecules *per se,* rather than from the medium in which the reaction was carried out. Thus the reactions catalyzed by alcohol dehydrogenase (Fisher *et al.,* '53), lactic dehydrogenase (Loewus *et al.,* '53), malic dehydrogenase (Loewus, Tchen, and Vennesland, '55), and testosterone dehydrogenase (Talalay *et al.,* '55) all involve a direct transfer of hydrogen between substrate and DPN.

In all these reactions, the reduced form of the substrate of the enzyme loses one atom of nonexchangeable hydrogen on oxidation by DPN. The experimental approach depends on this hydrogen's not exchanging with the medium. In general, two procedures are available for demonstrating the direct transfer of hydrogen between substrate and DPN. These may be illustrated for alcohol dehydrogenase by equations (2) and (3).

$$CH_3CH_2OH + DPN^+ \xrightleftharpoons[D_2O]{} CH_3CHO + DPNH + H^+ \qquad (2)$$

$$CH_3CD_2OH + DPN^+ \xrightleftharpoons[H_2O]{} CH_3CDO + DPND + H^+ \qquad (3)$$

In the first case, DPN may be reduced with unlabeled substrate in a medium of approximately 100% deuterium oxide. When this experiment was performed with yeast alcohol dehydrogenase, the isolated reduced DPN (DPNH) was found on analysis to contain no appreciable amount of nonexchangeable excess deuterium. In the second case, appropriately labeled reactants may be used in a medium of water. Thus, when DPN was reduced enzymically with α-dideuteroethanol as shown in equation (3), the acetaldehyde and the DPNH each contained one atom of nonexchangeable deuterium per molecule. The monodeutero-reduced DPN formed in this way has been designated DPND. The results just described are referred to as ''direct hydrogen transfer.'' The possibility that the hydrogen may be transferred by way of reduction and oxidation of the protein is considered unlikely (for a discussion of this question, see Vennesland and Westheimer, '54). For yeast alcohol dehydrogenase, such a possibility has been excluded by separate evidence obtained by Kaplan *et al.* ('52).

The use of deuterium has also permitted the demonstration that it is the carbonyl (i.e., not the enol) form of acetaldehyde that is reduced in the reaction catalyzed by alcohol dehydrogenase. Evidence for this conclusion was obtained by oxidizing DPNH enzymically with acetaldehyde in a medium of D_2O, and isolating the ethanol as the nitrobenzoate. The amount of deuterium found in the ethanol derivative was far below the one atom per molecule that would be required for reduction if the acetaldehyde were reduced as an enol. In a similar way, it has been shown for pyruvate, oxaloacetate, and androstenedione, that the form undergoing enzymic reduction is the keto compound, and not an enol (Loewus, Tchen, and Vennesland, '55; Talalay *et al.*, '55).

STERIC SPECIFICITY FOR DIPHOSPHOPYRIDINE NUCLEOTIDE

The steric specificity of the hydrogen transfer in the pyridine nucleotide dehydrogenase reactions has also been studied with the aid of deuterium. As already mentioned, the product formed by the chemical reduction of DPN in D_2O consists of a mixture of two diastereomers of monodeutero-DPNH (i.e., deuterium is introduced on either side of the plane of the nicotinamide ring). In the four enzyme reactions mentioned, the hydrogen transfer has been shown to be sterically specific for DPN, i.e., hydrogen is transferred to and from one side only of the plane of the nicotinamide ring. Three of the enzymes (alcohol and lactic and malic dehydrogenases) cause hydrogen transfer to and from the same side of the ring, but testosterone dehydrogenase causes the transfer to and from the opposite side of the plane of the ring. The steric specificity shown by alcohol dehydrogenase may be designated α, and the specificity shown by testosterone dehydrogenase may be designated β.

Table 1 gives a list of the enzymes for which direct hydrogen transfer has been demonstrated. The steric specificity for DPN is indicated for those cases in which it has been determined. The table includes a transhydrogenase from *Pseudomonas.* San Pietro *et al.* ('55) demonstrated that the reaction catalyzed by this enzyme involves a direct transfer of hydrogen from TPNH to DPN. They also showed that this transhydrogenase has the same steric specificity for DPN as the testosterone dehydrogenase. Since the two enzymes that show β stereospecificity for DPN were both obtained from *Pseudomonas,* this particular specificity might conceivably represent an idiosyncracy of the organism from which these two enzymes were obtained. Loewus therefore investigated the steric specificity of the alcohol dehydrogenase formed by the bacterial strain from which the testosterone dehydrogen-

TABLE 1

Demonstration of direct transfer of hydrogen

SUBSTRATE	ENZYME SOURCE	STERIC SPECIFICITY FOR DPN
Ethanol	Yeast Liver Wheat germ *Pseudomonas*	α α
L-Lactate	Muscle	α
L-Malate	Wheat germ Muscle	α
Testosterone	*Pseudomonas*	β
Triose phosphate	Yeast Muscle	
Glucose	Liver	
TPNH (transhydrogenase)	*Pseudomonas*	β

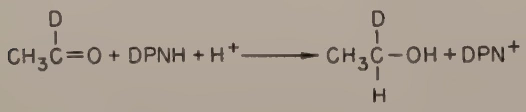
ase was prepared. The alcohol dehydrogenase from *Pseudomonas* showed α specificity for DPN, just as does yeast alcohol dehydrogenase.

The steric specificity of the alcohol dehydrogenase reaction for DPN was proved by the demonstration that all of the deuterium introduced into DPND by enzymic reduction with dideuteroethanol [eq. (3)] is removed on enzymic reoxidation with acetaldehyde. There is a hydrogen atom and a deuterium atom at position 4, and only the deuterium atom is removed. Such a result could be obtained only if the hydrogen transfer occurred to and from one side only of the ring, since some deuterium would otherwise necessarily remain in the reoxidized DPN.

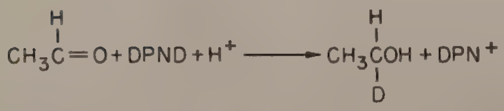
STERIC SPECIFICITY OF ALCOHOL DEHYDROGENASE FOR ETHANOL

Since pyridine nucleotide dehydrogenases having secondary alcohols as substrates (e.g., L-malate, L-lactate, testosterone) have long been known to show steric specificity for the substrate, it seemed possible that the reaction catalyzed by yeast alcohol dehydrogenase might also show steric specificity for the ethanol. This has been shown to be so by Loewus, Westheimer, and Vennesland ('53), by means of a procedure similar in principle to that used to demonstrate steric specificity for DPN.

Ethanol 1-*d* was prepared in two ways, as shown in equations (4) and (5).



$$CH_3\overset{D}{C}{=}O + DPNH + H^+ \longrightarrow CH_3\overset{D}{\underset{H}{C}}{-}OH + DPN^+ \qquad (4)$$

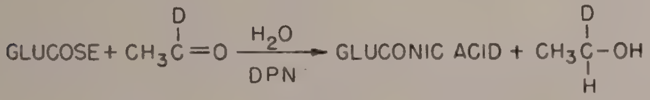


$$CH_3\overset{H}{C}{=}O + DPND + H^+ \longrightarrow CH_3\overset{H}{\underset{D}{C}}OH + DPN^+ \qquad (5)$$

In one procedure, deuteroacetaldehyde was reduced with unlabeled DPNH in the presence of yeast alcohol dehydrogenase.

In the other, unlabeled acetaldehyde was reduced with DPND prepared enzymically by reducing DPN with dideuteroethanol. If the reaction catalyzed by yeast alcohol dehydrogenase is sterically specific for ethanol, then the two α-monodeuteroethanols formed should be enantiomorphs. This was found to be the case. The two α-monodeuteroethanols were distinguished from each other by an examination of the products of their enzymic reoxidation. When ethanol formed by the reaction in equation (4) was oxidized by DPN$^+$, it yielded deuteroacetaldehyde and unlabeled DPNH. When ethanol formed by the reaction in equation (5) was oxidized, it transferred deuterium to form DPND and unlabeled acetaldehyde.

In the early experiments, the amount of the ethanol prepared was limited by the cost of the DPN. More recently, Levy and Loewus have devised a reasonably economical procedure for preparing one of the enantiomorphs of α-monodeuteroethanol in gram quantities. The procedure involves a coupling of the reactions catalyzed by glucose dehydrogenase and by alcohol dehydrogenase in the presence of a catalytic amount of DPN. The net coupled reaction is shown in equation (6).



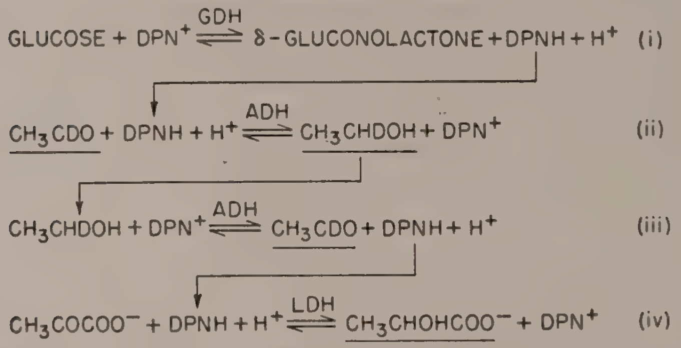
$$\text{GLUCOSE} + CH_3\overset{D}{\overset{|}{C}}=O \xrightarrow[\text{DPN}]{H_2O} \text{GLUCONIC ACID} + CH_3\overset{\overset{D}{|}}{\underset{\underset{H}{|}}{C}}-OH \qquad (6)$$

Strecker and Korkes ('52) have shown that the reaction catalyzed by liver glucose dehydrogenase involves an oxidation of β-glucopyranose to the δ-gluconolactone, which then undergoes nonenzymic hydrolysis to gluconic acid. In the coupled reaction, the function of glucose dehydrogenase is only to generate DPNH. Since the reaction is run in water, DPNH will be generated whether or not direct transfer of hydrogen occurs in the glucose dehydrogenase reaction; and the steric specificity of the over-all reaction for ethanol is determined only by the alcohol dehydrogenase.

Several grams of chemically prepared α-deuteroacetaldehyde was reduced by glucose according to reaction (6), and the ethanol 1-*d* was distilled out of the reaction mixture. A preliminary determination of the optical rotation of this ethanol gave $[\alpha]_D = +0.18°$. The individual steps in the procedure and the results of the deuterium analyses are summarized in table 2.

TABLE 2

Preparation and analysis of ethanol 1-d


GLUCOSE + DPN⁺ ⇌(GDH) δ-GLUCONOLACTONE + DPNH + H⁺ (i)
CH₃CDO + DPNH + H⁺ ⇌(ADH) CH₃CHDOH + DPN⁺ (ii)
CH₃CHDOH + DPN⁺ ⇌(ADH) CH₃CDO + DPNH + H⁺ (iii)
CH₃COCOO⁻ + DPNH + H⁺ ⇌(LDH) CH₃CHOHCOO⁻ + DPN⁺ (iv)

EQUATION NO.	COMPOUND ANALYZED	DILUTION FACTOR	ATOMS % EXCESS DEUTERIUM		ATOMS OF DEUTERIUM PER MOLECULE
			Found	Corrected	
2	Acetaldehyde	12.1	0.316	3.82	1.00
2	Ethanol [a]	44.1	0.229	10.1	0.91
3	Acetaldehyde	13.4	0.284	3.80	0.99
4	Lactate [a]	6.34	0.0035	0.022	0.003

[a] Derivatives not recrystallized.

Acetaldehyde was isolated as the dimedon derivative, ethanol as the paranitrobenzoate, and lactate as phenacyl lactate. GDH, glucose dehydrogenase; ADH, alcohol dehydrogenase; LDH, lactic dehydrogenase.

Equations (i) and (ii) in this table show the reactions involved in the synthesis of the ethanol, and equations (iii) and (iv) show the reactions involved in the analysis. The underlined substances were analyzed for deuterium. Reaction (iv) was introduced in order to determine the enantiomorphic purity of the ethanol with maximum precision. It had been shown previously that all of the deuterium introduced into DPND by enzymic reduction of DPN with dideuteroethanol is transferred in turn to lactate when the DPND is oxidized with pyruvate in the presence of lactic dehydrogenase. Such a transfer was carried out because the deuterium content of phenacyl lactate can be determined with much greater precision than the deuterium content of reduced DPN. Results of the deuterium analyses show that the monodeuteroethanol, the acetaldehyde from which it was prepared, and the acetaldehyde that is formed on enzymic reoxidation all contained approximately one atom of deuterium per molecule, whereas the lactate, and therefore the DPNH, contained essentially none.

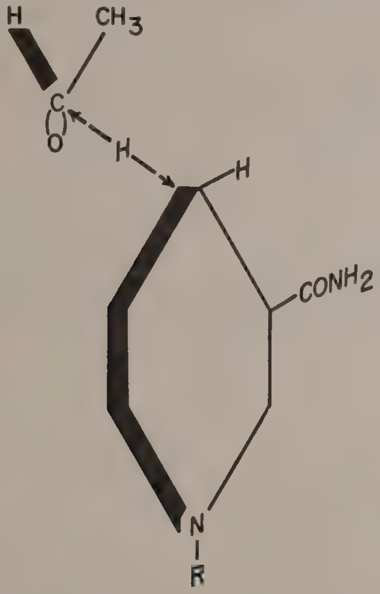
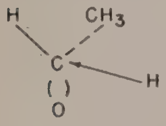


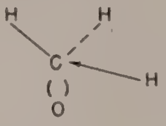
Figure 1

The geometry of the reaction catalyzed by alcohol dehydrogenase may be visualized with the aid of figure 1. This diagram does not represent absolute configuration, nor is it a picture of mechanism. The zinc, shown to be present in yeast alcohol dehydrogenase (Vallee and Hoch, '55), and the sulfhydryl groups (Barron, '51) have both been omitted, in view of the lack of decisive evidence for the nature of their specific role in the reaction.

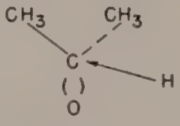
If it is assumed that figure 1 represents the geometry of the reaction normally catalyzed by yeast alcohol dehydrogenase, then the reaction's steric specificity for ethanol shows that no appreciable reaction occurs when the positions of the hydrogen atom and the methyl group of acetaldehyde are interchanged. It has been reported, however, that yeast alcohol dehydrogenase reacts to some extent, though relatively slowly, with both methanol (Barron and Levine, '52) and isopropanol (Barron and Levine, '52; Burton and Wilson, '53). The geometry of the reduction of acetaldehyde, formaldehyde, and acetone may be represented as in *a*, *b*, and *c*, respectively.



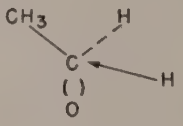
a



b



c



d

Reactions *b* and *c* are much slower than reaction *a*. However, the fact that they do occur at all suggests that there may be a measurable reaction when the acetaldehyde occupies the relative position shown in *d*. In other words, some racemization of the α-monodeuteroethanol may be expected. Such racemization would result in the appearance of deuterium in the lactate isolated in the experiment of table 2. The figure of 0.003 atom of deuterium per molecule of lactate may represent the sum of the racemization occurring in reactions (ii) and (iii) of table 2. This is a suggestion rather than a conclusion, in view of the possibility that the deuterium might be introduced into lactate by uncontrolled side reactions. It is also possible, but not probable, that the deuterium content of the lactate is not above that which is normal for unlabeled hydrogen, since the excess found was close to the lower limit of the amount detectable with the methods employed.

The full significance of the steric specificity of enzyme reactions involving tracer atoms was first discussed by Ogston ('48) in terms of what he called a "three-point landing." This terminology has led some to assume that a substrate must be "bound" to the enzyme at three different points in order to account for steric specificity. What is actually required to determine steric specificity is a particular orientation of the reacting molecules, and steric hindrances may be just as important as attractive forces for the determination of such orientation. The reaction catalyzed by alcohol dehydrogenase serves as a convenient example to illustrate this point. A substance such as ethanol or acetaldehyde may be bound to the enzyme through the oxygen. In addition, the methyl group may be held to the protein by van der Waals attractive forces, and the cohesive forces of the water may serve to "squeeze" the methyl group into a favorable site on the protein. The latter forces may not themselves be highly directed, however, and it is unlikely that the hydrogen atom attached to the carbonyl carbon atom can contribute to the binding. Under these circumstances, the orientation could be determined by steric repulsions that force the methyl group out of all possible positions except that in which it is properly oriented on the enzyme surface. Such an interpretation is borne out by the slower enzyme reaction with isopropanol than with ethanol.

THE OXIDATION OF ALDEHYDES

The experiments on hydrogen transfer have been extended by Loewus, Levy, and Vennesland ('55) to a study of the oxidation of aldehydes to acids. For both triosephosphate dehydrogenase and glucose dehydrogenase, it has been shown that these reactions, like the oxidation of alcohols, involve a direct transfer of hydrogen from substrate to DPN. Results of representative experiments are shown in table 3. The procedure involved, first, a reduction of DPN by the appropriate substrate and enzyme in a medium of approximately 100% deuterium oxide. When the reduction was complete, the enzyme was inactivated by heat and the D_2O was removed by lyophilization. The products were then dissolved in water and the DPNH was reoxidized by pyruvate in the presence of lactic dehydrogenase. Lactate was isolated as the phenacyl derivative and analyzed for deuterium, and the oxidized DPN was separated on a Dowex column, hydrolyzed to nicotinamide with DPNase, and the nicotinamide analyzed for deuterium. The table shows the results of the deuterium analyses of the lactate and nicotinamide. The value for lactate shows the amount of deuterium introduced into the α-4 position of the nicotinamide ring of DPN; that for nicotinamide shows all of the deuterium introduced anywhere else in the nicotinamide moiety. In the case of glucose dehydrogenase, a total of only about 0.02 of an atom of deuterium was introduced into DPN from the medium. It should be pointed out that, when the alcohol dehydrogenase reaction was run in D_2O and the DPNH was isolated and analyzed, as much as 0.03 of an atom of excess deuterium was also found in the reduced DPN. In this case, since the analyzed material was not pure, it was not possible to rule out the possibility that this deuterium was present in a contaminant of some sort. In either case, a small percentage of an atom of deuterium in no way alters the conclusions drawn from the experiments.

TABLE 3

Hydrogen transfer in oxidation of aldehydes

SUBSTRATE	ENZYME SOURCE	ATOMS OF DEUTERIUM/MOLECULE	
		Nicotinamide	Lactate [a]
Glucose	Liver	0.01	0.01
		0.01	0.01
Phosphoglyceraldehyde	Muscle	0.06	0.04
Phosphoglyceraldehyde	Yeast	0.06	0.07
Fructose diphosphate [b]	Yeast	0.06	0.13

[a] Analyzed as phenacyl lactate.

[b] Aldolase added.

In the experiments with triosephosphate dehydrogenase, the total amount of deuterium in the DPNH (i.e., the sum in lactate and nicotinamide) was well below one atom per molecule. The conclusion that the reaction involves a direct transfer of hydrogen is justified in this case also, but it should be pointed out that the exchange with the medium observed with this particular enzyme reaction is larger than that observed with any other enzymic reaction studied to date. The significance of this exchange has yet to be determined.

THE REACTION CATALYZED BY XANTHINE OXIDASE

In some enzymic reactions involving pyridine nucleotides, the substance that undergoes oxidation (or reduction) by the pyridine nucleotide does not contain extra nonexchangeable hydrogen in the reduced form. The cytochrome reductase and diaphorase reactions constitute such a group. These are flavoproteins. The reduced form of the isoalloxazine ring of the prosthetic group contains two extra hydrogen atoms, but this hydrogen is attached to nitrogen or oxygen and so would be expected to exchange very rapidly with the medium. Under these circumstances, the possibility of direct hydrogen transfer cannot be tested by the simple techniques used with the pyridine nucleotide dehydrogenases. The steric specificity of the reaction for the pyridine nucleotide can, however, be determined and, in special cases, the possibility of direct hydrogen transfer might also be tested. An investigation of xanthine oxidase to test for these factors is in progress.

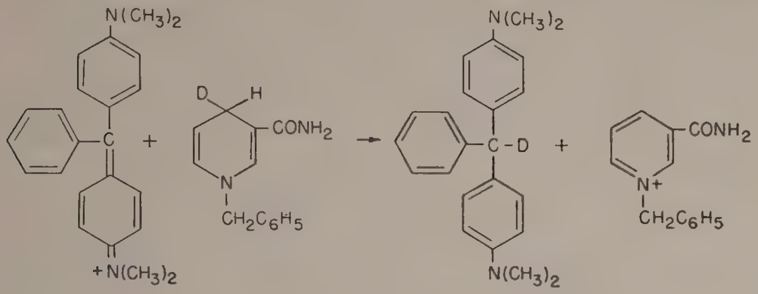
The reason for selecting this particular enzyme was that it catalyzes both reductions and oxidations of DPN by various reductants and oxidants. In preliminary experiments, Tchen and Loewus examined the enzymic reduction of DPN by α-deuteroacetaldehyde in the presence of xanthine oxidase. The system was coupled with lactic dehydrogenase, i.e., pyruvate was used as an oxidant for the DPNH, and DPN was added in only catalytic amounts. The lactate formed from the pyruvate was isolated as the phenacyl derivative and found, on analysis to contain 0.07 of an atom of excess deuterium per molecule. The presence of any excess deuterium at all must be considered significant in the sense that it shows that some of the α-hydrogen of acetaldehyde must have been transferred to DPN. If this transfer has occurred by way of the flavin, then the reduction of flavin by acetaldehyde must involve direct transfer of hydrogen also, though most of this hydrogen exchanges with the medium, as expected.

In another experiment, a sample of DPND prepared by enzymic reduction with dideuteroethanol was oxidized with xanthine oxidase, oxygen, and methylene blue in the presence of catalase. The oxidized DPN was hydrolyzed enzymically to nicotinamide, which was found on analysis to contain 0.65 atom of deuterium per molecule. Since the DPND contained one atom of deuterium per molecule, all of which was in the α position, it appears that xanthine oxidase is not sterically specific for DPN.

The results obtained with xanthine oxidase are preliminary. It should be borne in mind that DPN is probably not a natural substrate for xanthine oxidase, and that the reactions with DPN have very low turnover numbers. Furthermore, the enzyme preparations used in these experiments (prepared according to Mackler *et al.,* '54) exhibited a number of properties not anticipated. For example, the oxidation of DPNH by oxygen followed an autocatalytic course, i.e., a plot of the DPNH oxidized against time gave a sigmoid curve, as did a plot of the reaction between DPNH and cytochrome c. The reactions catalyzed by xanthine oxidase require further study.

MODEL REACTION

In connection with the studies of enzymic hydrogen transfer, it is of interest that Mauzerall and Westheimer ('55) have shown that the nonenzymic reduction of the dye malachite green by 1-benzyl dihydronicotinamide (a model compound for a pyridine nucleotide) likewise involves direct transfer of hydrogen from position 4 of the reduced nicotinamide ring to the oxidant, as shown in equation (7). In respect to hydrogen transfer, the model reaction resembles the enzymic reactions, except, of course, that steric specificity is lacking. As a consequence, there is nothing about the hydrogen transfer, *per se,* that inherently requires an enzyme.



(7)

GENERAL DISCUSSION

HAUROWITZ [3]: Dr. Vennesland, does it make any difference whether you use flavin protein with the metal and the metal free? It would be interesting to know whether this makes a difference in deuterium transfer.

VENNESLAND: The preparation that we have used is not free of metals. We have not determined what difference the presence of metals makes.

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MOLECULAR GEOMETRY IN ENZYME ACTION

DANIEL E. KOSHLAND, JR.
Biology Department, Brookhaven National Laboratory,
Upton, New York

ONE FIGURE

The last decade in biochemistry has seen the description of the properties of an increasingly large number of pure enzymes. Knowledge of such factors as molecular weights, *p*H optima, and kinetic constants has become available for an impressive array of these protein catalysts. The basic explanation for the catalytic action, however, remains obscure. Various suggestions have been offered based on mechanical and chemical analogies. Some seem reasonable, but it is not known whether they make an appreciable contribution to the catalysis; others seem unreasonable, but specific arguments against them cannot be cited. It is illuminating to find that a hypothesis which is "obviously true" to one biochemist is "clearly preposterous" to the next. In this paper, a few steps toward evaluating the catalytic power of enzymes will be described.

GOAL OF QUANTITATIVE EVALUATION

It is most likely that enzyme action is a combination of a number of properties of which a few may predominate. The goal will be to assign the proper weight to each of the suggested sources of catalytic action and thus arrive at the enzymic rate. Although there is no assurance that this will be a linear function, a linear equation will be helpful to illustrate the method. If A, B, C, etc., in equation (1) represent

$$V = a[A] + b[B] + c[C] + d[D] + \ . \ . \ . \qquad (1)$$

a function for the particular source of enzyme action, e.g., the collision frequency hypothesis, the energy transfer theory, the strain theory, a van der Waals attraction theory, etc., the parameters *a, b, c,* represent the quantitative contribution of each of these terms to the over-all action of the enzyme. The ultimate goal is (1) to be able to assign numerical values to these parameters from the known properties of the substrates and the protein molecule and (2) to have the calculation, based on these parameters, accurately predict the velocity of the enzyme reaction.

Phrased this way, realization of the goal is distant, particularly in view of inadequate knowledge of protein structure. It is valuable, however, in indicating a direction, i.e., the separate systematic evaluation of each possible source of catalysis. Although, at the moment, the rate of a particular enzymic reaction cannot be predicted, maximum possible values may be assigned to the parameters. These will indicate whether the postulated mechanism is totally inadequate to explain enzyme action, a possible partial factor, or capable of explaining the entire enzymic velocity.

COLLISION FREQUENCY THEORY

One of the most widely accepted explanations of enzyme catalysis is based on the collision frequency in the ternary complex formed between enzyme and two substrates. Probably the simplest statement of this hypothesis is given by Michaelis ('46) with respect to the oxidation-reduction reaction: "When a molecule of one of these two substances collides with a molecule of the other in solution, the chance of an electron-transfer during the short time of collision is nil; but when these two molecules are held close together in appropriate juxtaposition and orientation with respect to each other they remain in the spatial arrangement for a long time during which an electron transfer may occur once in a while." These arguments would also be pertinent for nonoxidative reactions such as group transfers and aldol condensations.

This statement implies that: (1) the frequency of collisions between the substrates is increased, and (2) the orientation of the substrates with respect to each other is improved. The first of these, which shall be called for convenience "the collision frequency hypotheses," can be examined quantitatively in the following way.

The rate of the nonenzymic reaction shown in equation (2)

$$A + B \rightarrow \mathrm{P} + Q \tag{2}$$

can be expressed by the usual rate law

$$\frac{d[\mathrm{P}]}{dt} = k[A][B]. \tag{3}$$

In the collision frequency hypothesis, it is not assumed that the total number of collisions of A with all molecules increases, but only the number with the second substrate B. Hence it follows that the rate on the enzyme surface occurs with the same rate law and the same rate constant as in the solution as a whole. Thus the rate will be

$$\frac{d[\mathrm{P}]_E}{dt} = k[A]_E[B]_E \tag{4}$$

where $[\mathrm{P}]_E$, $[A]_E$, and $[B]_E$ refer to the concentrations of these substances on the enzyme surface. If these concentration terms can be evaluated, it may be possible to indicate quantitatively how much this type of catalysis contributes to the enzyme action. The approach taken was to obtain the maximum value for the rate based on the collision frequency hypothesis and then to compare this with experimental values. To do this it was assumed that (1) $[A]_E$ and $[B]_E$ are equal to the concentrations of pure A and pure B respectively, (2) the rates of absorption of A and B and of desorption of P and Q from the enzyme surface are infinitely great, (3) the active site of the enzyme is saturated with A and B, and (4) the total volume available to P at each active site on the enzyme surface is equal to that occupied by the sum of the volumes of pure A and pure B. The resultant ratio of the rates of product formation for the nonenzymic reaction (V_0) and for the enzymic reaction (V_E) is

$$\frac{V_E}{V_0} = \frac{10^3 D_A D_B [E]}{M_A M_B [A][B]}\left(\frac{M_A}{D_A} + \frac{M_B}{D_B}\right) \tag{5}$$

where D represents density in grams per cubic centimeter of the substrate indicated in subscript, M, molecular weights of the substrates, $[E]$, enzyme concentration in equivalents per liter, i.e., active sites per mole times moles per liter, and $[A]$ and $[B]$ represent the concentrations of substrates in the solution as a whole in moles per liter.

The values of the rates with pure enzymes of known molecular weight were obtained from the literature and are shown in the fifth column of table 1. The rates of the nonenzymic reactions were obtained from suitable model systems in sterilized solutions incubated for several days. Concentrations in these control runs were usually higher than the substrate levels recorded in order to raise the level of detection. "V_0 observed," however, was corrected to the substrate levels shown in column 2 by calculation from the rate constant. The theoretical ratio V_E/V_0 was obtained from equation (5) by inserting the concentrations of $[E_T]$, $[A]$, and $[B]$ used in the enzymic reaction.

The wide discrepancy between the observed V_E/V_0 ratio and that calculated from the collision frequency theory (see table 1) shows that the latter is completely inadequate as an explanation for enzyme catalysis. The juxtaposition of pure A and pure B would certainly increase the total collisions of A with B if enzyme were present in high concentrations; but with the low concentrations of enzyme normally encountered, it is seen that the number of collisions between substrates on the enzyme surface is often only slightly greater or even smaller than those occurring randomly in solution. The detailed assumptions were crude in some cases, e.g., the density of adenosine triphosphate (ATP) was assumed to be equal to that of KH_2PO_4, but these would involve small corrections in a discrepancy that goes as high as 10^{11}. The theoretical assumptions were chosen to give a maximum rate, e.g., the actual rates of absorption of substrates and desorption of products may be measurable, and hence a disagreement with these assumptions would only magnify the discrepancy between theory and experiment.

TABLE 1

Comparison of observed enzymic rates with those calculated by collision frequency theory

ENZYME	SUBSTRATE AND CONCENTRATION	$[E_T]$	OBSERVED		V_E/V_0 CALCULATED FROM THEORY	$\frac{V_E/V_0 \text{ OBSERVED}}{V_E/V_0 \text{ THEORY}}$
			V_0	V_E		
		equiv./l [a]	*(moles/l)/min*			
Hexokinase	0.0003 M Glucose 0.002 M ATP	10^{-7}	$< 1 \times 10^{-13}$	1.3×10^{-3} [b]	1.8	$> 7 \times 10^{9}$
Phosphorylase	0.016 M Glucose 1-phosphate 10^{-5} M Glycogen	6×10^{-8}	$< 5 \times 10^{-15}$	1.6×10^{-3} [c]	2.5	$> 1.4 \times 10^{11}$
Alcohol dehydrogenase	0.0004 M DPN 0.04 M Ethanol	4×10^{-7}	$< 6 \times 10^{-12}$	2.7×10^{-3} [d]	0.5	$> 9 \times 10^{8}$
Creatine kinase	0.024 M Creatine 0.004 M ATP	3×10^{-9}	$< 3 \times 10^{-9}$	4×10^{-5} [e]	0.5×10^{-3}	$> 2.7 \times 10^{7}$

[a] Number of moles per liter multiplied by number of active sites per mole.

[b] Colowick, '51.

[c] Illingworth, '53.

[d] Velick, '54.

[e] Kuby *et al.*, '54.

This does not mean, of course, that no advantage is gained by the ternary complex with enzyme. The close juxtaposition of substrates is seen to compensate in whole or in large part for the very low concentration of enzyme and this value will be even more marked when, for example, a third substrate or activators such as metal ions must be present in addition to the two substrates. This calculation emphasizes the need for other theories of catalysis and gives a quantitative figure for the amount of added catalytic action required even after the substrates are held in close juxtaposition by the enzymes.

ENERGY TRANSFER THEORY

The observation that most enzymic reactions have lower activation energies than their chemical analogs led naturally to the hypothesis that the enzyme might be activating the substrate by direct transfer of energy. Many elegant formulas have been derived relating activation energies to degrees of freedom and numbers of quadratic terms (Moelwyn-Hughes, '50) but the transfer process might be depicted roughly as follows: The enzyme attracts and holds substrate *A* to the protein surface by attracting several functional groups in the substrate to several active spots on the enzyme. The parts of the enzyme that are constantly undergoing vibration, rotation, and translation stretch or compress the substrate and thus impart added energy to it. This energy "activates" the substrate. Ultimately, of course, the thermal energy must be restored to the enzyme from the solvent or products, but the multiple binding to the large polymer presumably increases the effectiveness of the energy transfer.

A test of this hypothesis was obtained by considering the hydrolytic action of an enzyme on a series of substrates of general formula R-O-Q. The enzyme, according to this hypothesis, stretches or compresses the ROQ molecule, thereby providing the additional activation energy required for hydrolysis to the products ROH and QOH. Let us assume that a series of substrates can be prepared in which R is held constant but Q is varied so that the ease of breaking of the

Q-O bond in the nonenzymic reaction is less than, equal to, and greater than that of the *R*-O bond. If the enzyme simply provides energy to the *ROQ* molecule, *R*-O bond cleavage should be expected in the first case, a mixture in the second, and *Q*-O cleavage in the third.

Such a set of substrates is available for the hydrolyses catalyzed by alkaline phosphatase. If the phosphate esters under consideration are glucosyl phosphate, butyl phosphate, and phenyl phosphate, knowledge of the hydrolytic action of the corresponding halides and of phosphate esters indicates that the glycosyl-oxygen bond is far more readily split than the phosphorus-oxygen bond, the phenyl-oxygen bond is far more difficult to split than the phosphorus-oxygen bond, and the butyl-oxygen bond is of comparable value. According to this theory, carbon-oxygen cleavage should be expected when the enzyme acts on glucose 1-phosphate, phosphorus-oxygen in phenyl phosphate, and either or both in butyl phosphate.

TABLE 2

Cleavage point in hydrolyses catalyzed by alkaline phosphatase

PHOSPHATE ESTER	O^{18} CONTENT OF H_2O	O^{18} CONTENT OF INORGANIC PHOSPHATE LIBERATED		
		Calculated for C-O cleavage	Calculated for P-O cleavage	Observed
	atom % excess			
Glucosyl	0.939	0.00	0.24	0.25 [a]
Butyl	1.23	0.00	0.30	0.25 [b]
Phenyl	1.37	0.00	0.34	0.34 [b]

[a] Cohn, '49.

[b] Stein and Koshland, '52, and unpublished.

The results of actual experiments with H_2O^{18} to determine the point of cleavage are shown in table 2. Phosphorus-oxygen splitting was observed in every case.

The results indicate that the energy transfer theory must be a minor factor in the hydrolysis. Unlike the analysis in the collision theory, no precise mathematical value can be given to the limit of contribution of such a mechanism but it, apparently, would have to be very small to have no detectable effect over the range of glucosyl-oxygen to phenyl-oxygen bonds.

It can be argued that the energy transfer occurs at a specific point in the substrate molecule and in essence ''knocks out'' the phosphate group. This is essentially what occurs in ''hot'' atom chemistry. However, the requirements for such an effect necessitate a free atom of high kinetic energy. Since the atoms in the protein are bound together by covalent bonds, they can impart energies only of the order of activation energies rather than of bond dissociation energies.

CATALYTIC GROUP ORIENTATION THEORY

The ''catalytic group orientation'' theory of enzyme action will be taken to mean that the catalytic action of the enzyme is derived from the precise orientation of groups that polarize the electrons in the substrates at the points of bond breaking and bond formation. It has been known, of course, for a long time that a precise geometry is required in many reactions for the fit of a substrate to an enzyme surface. This fit, however, refers to the affinity of the substrates for the enzyme surface and does not explain their increased reactivity with each other after they become bound. In the theory referred to here, an attempt is made to explain the latter.

Before listing the evidence for the theory, a specific model may clarify what is meant; for this purpose, the action of β-amylase will be considered. The enzyme surface can be visualized schematically as shown in figure 1. The fact that the reaction causes inversion of configuration indicates that it proceeds by a single displacement mechanism of the type shown, i.e., by direct attack of the water on the carbon atom (Koshland, '53, '54). The reaction then involves the electron-donor properties of the water molecule and the readiness with which the electrons can be withdrawn toward oxygen from the maltosyl carbon atom. From physical organic chemistry, it is known that hydroxide ion is a much more powerful electron donor and a more powerful nucleophilic reagent than water. A basic group, *B*, on the enzyme surface that would attract a proton and partially or completely convert water to hydroxide ion would, therefore, probably increase the rate of reaction. Likewise, an acidic group, A^+, which would attract the electrons at the maltosyl oxygen and hence break the C-O bond, will also accelerate the reaction. Clearly, however, these two groups must be spaced precisely, for the oxygen of the water must readily make a bond with the carbon of the polysaccharide chain. Thus the oxygen of the water must be approximately the length of a C-O bond from the maltosyl carbon and the oxygen-carbon bond in the polysaccharide is, of course, of an equivalently definite length. Similar arguments can be used for a double displacement mechanism, in which case the nucleophilic site on the enzyme has the role of the basic group.

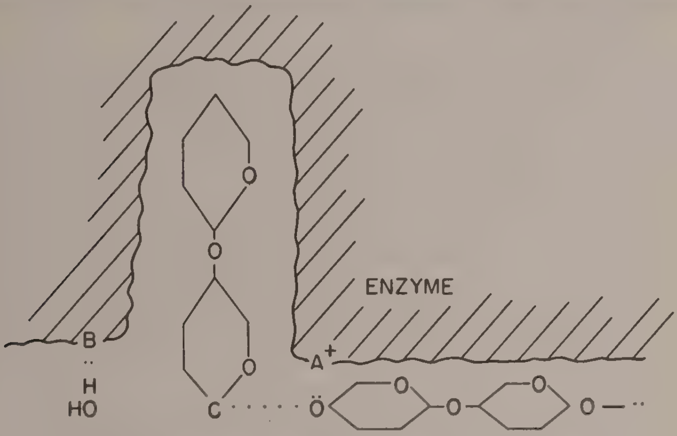

Fig. 1 Schematic enzyme surface for β-amylase.

Evidence that the spacing of catalytic groups has a bearing on enzyme action can be adduced from heterogeneous catalysis, homogeneous catalysis, and the actions of the enzymes themselves.

Heterogeneous catalysis. The early suggestions that there is a geometric or lattice spacing factor in heterogeneous catalysis was made by Langmuir ('21) and the first evidence was probably obtained by Adkins ('22). Since then a number of phenomena have been explained by this spacing, of which two examples may be given.

1. It can be calculated from bond angles and bond distances that the hydrogenation of ethylene on nickel surfaces should occur more readily with 3.50 A spacings than with 2.47 A spacings. The (111) plane of nickel contains only 2.47 A spacings whereas the (110) plane contains both 2.47 A and 3.50 A spacings. There should, therefore, be higher activity on the (110) plane; this, indeed, proved to be the case (Twigg and Rideal, '40; Beeck *et al.*, '40).

2. In the dehydrogenation of cyclohexane, similar theoretical considerations indicate a symmetrical lattice of 2.69 A spacing is ideal for the catalytic action. Platinum catalysts oriented to expose preferentially (110) planes can be prepared, whereas the nonoriented films contain much larger exposures of (111) planes. Since only the (111) planes show the proper symmetry and spacing, the nonoriented films should be more active than the oriented and they are by a factor of 10 (Balandin, '29; Trapnell, '51; Beeck and Ritchie, '50).

Thus there is strong evidence that the spacing in heterogeneous catalysis contributes importantly to the catalytic action. In Adkin's case it was even postulated to be largely responsible for the shift from acetone to ethylene formation in the decomposition of ethyl acetate.

Homogeneous catalysis. The chemical analogs of enzymic reactions all require the simultaneous occurrence of two antithetical processes. If one substance is reduced, another is oxidized. If one group leaves with a pair of unshared electrons, another enters to form a shared electron bond. The reagents that will catalyze one part of the process will, therefore, inhibit the other. The best known mechanisms occur in the substitution reactions shown in equation (6). An acidic group pulling the electrons in X out of the BX bond

$$YH + BX \rightarrow \overset{\mathrm{H}}{Y}: \ldots B \ldots :X \rightarrow BY + HX \qquad (6)$$

will accelerate the departure of the *X* group. A basic group removing H will increase the electron-sharing tendencies of *Y*. Clearly, the reaction should be accelerated by strong base and strong acid, but the simultaneous addition of these two reagents merely results in their neutralization. With weaker reagents, however, an acidic group ''pulling'' and a basic group ''pushing'' would seem to accelerate the reaction; Lowry and coworkers (Lowry, '25; Lowry and Richards, '25) found that both an acid and a base were required in the mutarotation of glucose. Swain has extended and generalized this work suggesting that all displacements occur by such a termolecular mechanism (Swain, '53; Swain and Eddy, '48). This has been opposed by some workers and the universality of the termolecular mechanism is still in dispute. The fact that some reactions under unfavorable solvent conditions require it whereas others under more favorable conditions may not suggest, at the least, that this represents a powerful form of catalysis, which may be superfluous under certain circumstances.

The combined action of acids and bases would appear, therefore, to be ideally suited for enzyme catalysis, since the protein contains acidic and basic groups and these groups can be held apart in space to prevent mutual interaction. This ''polyfunctional'' catalysis was elegantly demonstrated by Swain in what may be considered to be an enzyme model, i.e., he showed that 2-hydroxypyridine catalyzed the mutarotation of glucose far more effectively than equal concentrations of pyridine and phenol (Swain and Brown, '52a, b). Furthermore, 3- and 4-hydroxypyridine were ineffective as polyfunctional catalysts. Thus a single molecule with both acidic and basic sites in precisely the proper orientation was found to be a highly effective catalyst.

pH Dependence. In his studies on acetyl cholinesterase, Wilson ('54) was able to show that the observed *p*H dependence of his enzymic activity was explained by the requirement for a basic group and an acidic group. Moreover, he was able to show by competitive inhibition experiments with prostigmine that the acidic group was involved primarily in the hydrolysis and not the binding. Inhibition experiments with phosphorylating agents, moreover, indicated that the basic group was responsible for the attack on the acyl carbon of one of the substrates. Myrbäck ('47) and others (Myrbäck and Björklund, '52; Myrbäck and Persson, '53) have shown similar *p*H-activity curves which, although less specifically tailored to the enzyme action, suggest the requirement of both acid and base in the enzymic catalyses. These cases do not establish that the acidic and basic groups are both required for reaction after absorption to the enzyme rather than or in addition to binding, but they are certainly consistent with the theory. Frieden and Alberty ('55) have demonstrated by elegant *p*H-activity curves, that fumarase requires both a basic and an acidic group. Since maximal initial velocities are used, the groups are evidently required for catalysis and not merely binding.

Correlation of specificity and bond breaking. In a correlation of the point of bond cleavage with the specificity requirements of enzymes, Koshland and Stein ('54) were able to suggest a rule to predict the point of bond breaking. On the assumption that (1) the reactions occurred by displacement mechanisms, and (2) a precise orientation of catalytic groups was required for enzyme action, it was deduced that enzymes hydrolyzing ROQ that show high specificity for R and low specificity for Q will split the R-O bond. Examples that agreed with the rule were reported, and it has been shown that β-glucosidase also supports the generalization (S. S. Springhorn and Koshland, unpublished). The evidence for this rule can, therefore, be considered as support for the assumptions made in its derivation, and hence for the orientation of catalytic groups as a basis for enzyme catalysis.

Quantitative evaluation. Quantitative evaluation of this theory of enzyme action is possible by a method similar to that used in the evaluation of the collision frequency hypothesis. This evaluation is less clear cut than might be desired because of deficiencies in the physical organic data, but rough calculations indicate, for example, that the maximum rates (turnover numbers) observed in the esteratic action of trypsin, chymotrypsin, carboxypeptidase, and acetyl cholinesterase are compatible with extrapolations from the ester hydrolysis rates catalyzed by hydrogen and hydroxyl ions (Koshland, unpublished).

IMPLICATIONS OF THE CATALYTIC GROUP ORIENTATION THEORY OF ENZYME ACTION

Other possible sources must and will be evaluated before any definitive value for the role of the catalytic group orientation theory can be assigned. It is sufficiently attractive, however, that it may be worth while to consider at least one implication, which follows, if it is indeed a major factor in enzyme action.

That implication is that there is no requirement for large size in the catalytic molecule. It has been felt by many that, since proteins are large molecules, the size must be an inherent part of the catalytic action. It has been seen that the energy transfer theory that would account for the size of the protein molecule does not seem to apply to enzyme action. Moreover, van der Waals forces that would also provide a rationale for large size would seem to be of minor influence since the collision theory analysis indicates that the mere binding of substrates to the enzyme surface does not explain enzyme action. The orientation of catalytic groups, however, requires only that the size of the enzyme be of the order of the distance between forming and breaking bonds. This theory, therefore, provides no inherent necessity of high molecular weights for the catalyst molecules.

Actually, enzymes differ enormously in size from ribonuclease of molecular weight of ca. 13,000 to enzymes like actomyosin of molecular weights in the millions. Nor does the molecular weight parallel the activity for even a particular enzyme. Thus Gergely ('53) found that the fragment of myosin split off by trypsin digestion had two to three times the activity of the original molecule. It still might be argued that a minimum size of the order of the ribonuclease molecule is required even though further increase in size will not necessarily increase the catalytic activity. Even this seems doubtful in view of Perlmann's results ('54), which show that a dialyzable portion of the pepsin molecule still retains enzymic activity. The conditions of this dialysis (complete dialysis in 24 hours at 5°C.) suggest that the maximum molecular weight must be a few thousand and may even be considerably smaller. Thus the available data on enzymes show neither a parallelism between molecular weight and enzyme activity nor a requirement of high molecular weight for catalytic action.

The fact that enzyme action can be obtained from small molecules is in no way incompatible with the observed high molecular weight of most enzymes. The reason for the latter is speculative at the moment, but it could, of course, follow from evolutionary as well as catalytic considerations. The folding of the protein may well require high molecular weights in some cases, e.g., the catalytic groups may be across two chains, or an adjacent chain may be required to maintain the orientation of the active site. The point made here is simply that it is clearly consistent with this theory that a small fragment of the enzyme can still retain activity and that small molecules can duplicate enzyme action.

SUMMARY

In an attempt to evaluate the catalytic powers of enzymes, three theories of enzyme action have been examined. Comparison of theoretical and observed ratios of enzymic velocity and nonenzymic velocity for varied sample reactions shows that the collision frequency hypothesis cannot account for enzyme catalysis. The cleavage points of phosphate esters during enzymic hydrolyses indicate that the energy transfer theory of enzyme action is likewise a minor or negligible factor in enzyme catalysis. Evidence from heterogeneous catalysis, homogeneous catalysis, and from the action of the enzymes themselves, however, suggest that the precise orien-

tation of catalytic groups at the points of bond formation and bond cleavage makes an important contribution to the enzyme action. The latter theory implies that there is no necessity for high molecular weight in the enzyme molecule.

GENERAL DISCUSSION

Perlmann[1]: Since Dr. Koshland referred to our work on the formation of dialyzable active fragments from pepsin, I shall attempt to summarize the results of our experiments briefly. If pepsin solutions in the *p*H range of 4.6–5.6 are incubated at 37°C., portions withdrawn after 24 or 48 hours and these samples dialyzed, enzymically active fragments are found in the dialyzate. The dialyzable material represents 10–20% of the total protein. If tested against hemoglobin, it has an activity per unit of nitrogen of 1–5% relative to that of the intact enzyme. If, however, the activity of the dialyzable fragments is tested with the synthetic substrate, acetyl 1-phenylalanyl diiodotyrosine, it is about 75% of the value of the intact protein.

The dialyzable material is inhomogeneous and can be separated chromatographically on Dowex-50, 4% cross linked, into four or five fractions, two of which are active. The molecular weight of these materials is unknown.

Since it has been demonstrated by Sanger that pepsin hydrolyzes a variety of peptide bonds, it would appear from our experiments that the dialyzable, enzymically active fragments represent a portion of the original protein with a specificity preferentially directed toward peptide linkages adjacent to an aromatic amino acid.

In addition to the results just mentioned, we found that *Bacillus subtilis* contains an enzyme that has pepsin-like properties. It hydrolyzes both hemoglobin and acetyl 1-phenylalanyl diiodotyrosine at *p*H 2.0 but not at values more alkaline than *p*H 4.0. As in pepsin, this enzyme, if incubated at 37°C., undergoes autodigestion with the formation of enzymically active, dialyzable fragments. To my knowledge, these findings represent the first experimental demonstration of a pepsin-like enzyme in bacteria.

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[1] Gertrude E. Perlmann, Rockefeller Institute for Medical Research, New York City.

I should like to ask Dr. Koshland two questions:

1. If a phosphatase hydrolyzes an oxygen-phosphorus bond, e.g., that of phosphoserine, and if it is assumed that this phosphoamino acid is present in an unfolded peptide chain, will an adjacent dicarboxylic acid or a hydroxyamino acid or any of the structures such as those that follow influence the enzymic activity of the phosphatase, e.g., would it change the point of bond splitting?

2. Can you formulate what may happen in the partial inactivation of an enzyme?

KOSHLAND: An adjacent group might change the point of splitting in a nonenzymic reaction via a ''neighboring group'' effect, but I should not expect it to change the mechanism of the enzyme action. Therefore, if the phosphoprotein is a substrate for alkaline phosphatase, I should expect phosphorus-oxygen splitting. Added adjacent groups might increase or decrease the enzymic reaction rate but I should be very surprised if they caused a change from phosphorus-oxygen to carbon-oxygen cleavage.

Now the second question is a very interesting one. It seems possible that part of the enzyme not precisely at the active site is altered, with consequent change in, but not destruction of, the enzyme action. For example, I have often worried about the large ATPase activity in aged mitochondria because it seems so inefficient to have large ATPase action at the site of ATP production. Perhaps the enzyme is a kinase which is partially altered and becomes an ATPase.

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MOLECULAR GEOMETRY IN ENZYME ACTION

(INVITED DISCUSSION)

A. L. DOUNCE
Department of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, New York

FOUR FIGURES

Dr. Koshland has given an elegant presentation of his theory of group orientation. I have tried to express the same theory, if I understand it correctly, in a more qualitative manner in an article now in press for *Enzymologia*. By way of pure speculation, some guesses based on the new theory will be made as to the possible nature of a few prosthetic groups or active centers of some enzymes.

Succinic acid is rather inert from the standpoint of organic chemistry, but in the light of the orientation theory, it might be possible to understand how an enzyme could overcome this inertness (fig. 1).

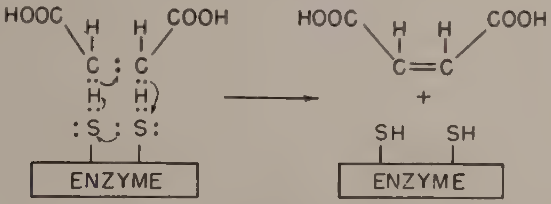

Figure 1

The substrate (succinic acid) is held to the enzyme by two basic groups (not shown) and there is a prosthetic group assumed to contain two sulfur atoms, which is held in juxtaposition to the succinic acid molecule. The mechanism of the dehydrogenation is obvious from the figure. Singer and Kearney ('55) found α-lipoic acid and iron in highly purified succinic dehydrogenase but could not decide whether or not the lipoic acid was an impurity.[1] However, Singer *et al.* ('55) had found that the purified enzyme was strongly inhibited by all types of —SH reagents. The type of mechanism proposed here could conceivably work with lipoic acid as the first prosthetic group to receive the hydrogen atoms from the substrate.

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[1] At a meeting in Detroit (International Symposium on Enzymes, Nov. 1–3, 1955), Singer stated that the enzyme is an iron flavoprotein, but failed to elucidate the role of –SH groups.

Another problem is the question of the chemical nature of the active centers of proteolytic enzymes. For a metal-activated hydrolase, a hypothetical mechanism such as shown in figure 2 may be written.

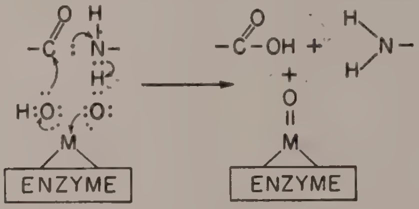

Figure 2

Here a metal atom bearing two -OH groups is assumed to be coordinated with metal-binding atoms on the enzyme molecule, and some kind of substrate-binding groups (not shown) are also present on the enzyme molecule, which will bring the peptide into proper juxtaposition with this metal dihydroxide. Then if the proper electronic shifts are assumed, hydrolysis results and the metal is left as an oxide that might be expected to become hydrated (without catalysis) in order to regenerate the original material.

However, a metal does not necessarily have to be used. It would seem that a hydrated aldehyde group would also be acceptable. Dr. Fraenkel-Conrat made some statements (this symposium) about stabilizing ordinarily unstable organic groups by attaching them to protein. Instead of metal, there might be the structure shown in figure 3. Now, by the same sort of reasoning, the end product would be the aldehyde, which then should spontaneously react with water to regenerate the aldehyde hydrate, as shown by the formulas.

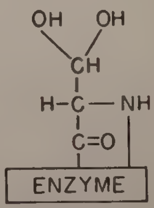

Figure 3

Could such a mechanism possibly explain the results of the inactivation of trypsin by diisopropylfluorophosphate (DFP)? If the —OH groups of the hypothetical aldehyde hydrate are more reactive than other —OH groups in the trypsin molecule, a double compound containing two DFP's might be formed. If one of the DFP's then reacted with histidine, the result might be the type of interchange shown in figure 4. One of the DFP molecules is transferred onto the nitrogen and is replaced by hydrogen. The nitrogen would be oxidized at the expense of the aldehyde group, and then there would be only one DFP attached to an amino acid, which would finally be isolated as serine.

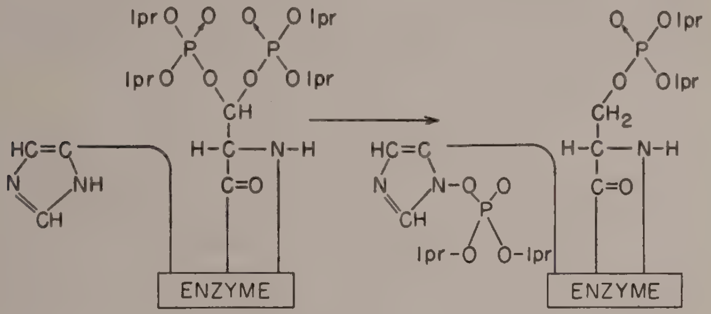

Figure 4

I have tried to apply this same type of thinking to catalase, by assuming that a second hematin group exists in just the right spatial configuration relative to the first one, so that the two hydrogen peroxide molecules that react are held close together by being bound to the iron atoms of these hematin groups. There might be four hematins in a square, but a good deal of calculation would be necessary to determine the feasibility of such an arrangement.

Some work bearing on this point has been done in our laboratory. In lyophilizing catalase under the proper conditions, something apparently is done to the geometry of the prosthetic group which allows the hematin iron atoms to be reduced by sodium hydrosulfite. Previous to lyophilization, these iron atoms are not reducible by hydrosulfite. The structure around the iron in catalase is analogous to that in methemoglobin. There is an —OH group attached to each iron atom and this —OH can be replaced by peroxide, chloride, acetate, or certain other ions. Lyophilized catalase can certainly be reduced to the extent of 90%, and yet the activity is diminished only by one-third to one-half. The spectrum, before adding any reducing agent, is indistinguishable from the spectrum of the original material. It is now concluded that slight changes in the geometry of the prosthetic groups alter enzymic activity but do not necessarily abolish it. The experimental work in question was published in 1950.

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THE ACTIVE GROUPS OF PEPSIN

(INVITED DISCUSSION)

ROGER M. HERRIOTT
Department of Biochemistry, The Johns Hopkins University,
School of Hygiene and Public Health,
Baltimore, Maryland

ONE FIGURE

Pepsin is of interest for many reasons, but attention is directed here to it as representing those enzymes that probably contain no prosthetic (nonamino acid) groups. Its proteolytic activity must, therefore, be attributed to the arrangement of its amino acids in the peptide chain and/or to the folding of the chain into a specific structure. This specific folding needs emphasis for denaturation of the protein, which involves an unfolding and not a change in amino acid arrangement and is accompanied by a complete destruction of the peptic activity. Dr. Perlmann's interesting results ('54) require special consideration and are considered by her elsewhere in this symposium.

It has been of some interest to determine the nature and number of protein structures, on the surface of this enzyme, that contribute to its activity. Pepsin has 36 carboxyl, 3–4 amino, and 17 tyrosine phenol groups per molecule (Northrop *et al.*, '48). It was an obvious reaction to wonder which and how many of these various protein groups are necessary for enzymic activity. This problem was examined by applying relatively specific reagents under controlled conditions and determining how the enzyme activity changed as the various protein structures were modified. In this work, no distinction was made between protein structures that bind the substrate and those that are catalytically important. Such a separation of function was observed in chymotrypsin by Doherty and Vaslow ('52), but there is no pertinent data for pepsin.

Three different chemical reagents have been applied to solutions of pepsin, and they attack the protein at different points. Sulfur mustard attacks the protein carboxyl groups (Herriott *et al.*, '46); iodine substitutes into the benzenoid moiety of tyrosine (Herriott, '37, '41, '47); and ketene reacts with the tyrosine phenol group and also with the free amino group (Herriott, '35). Since the action of ketene on the amino group has no effect on the activity (Herriott and Northrop, '34), it need not be given consideration. It is well known, of course, that iodine substituted into benzenoid moiety of tyrosine has a pronounced effect on the ionization of the phenolic group. Even though ketene and iodine attack tyrosine residue at different points, the effect is probably due to the effect on the phenolic group.

Figure 1 shows the effect of the three reagents mentioned on the peptic activity. Several conclusions may be reached from an examination of these results. In the first place, it is clear that modification of either the carboxyl or the phenolic groups resulted in a loss of enzymic activity; hence, both structures on this protein are probably needed for full peptic activity. It is important, however, to determine if possible the number of carboxyl and tyrosine moieties which are essential to the enzyme. If only sulfur mustard reaction is considered, it may be seen (fig. 1) that a drop of 0.3 of a log, i.e., a 50% drop in activity, involves an average coverage of only 3.5 carboxyl groups per molecule of pepsin or only 10% of the total number.

The next point of interest is that the semilogarithmic plot is linear over about 90–95% of the inactivation reaction. Despite a conclusion to the contrary noted in the McCollum-Pratt Symposium (Herriott, '54), this reaction appears to be quite similar to the dosage type plot of radiobiologists. It is necessary only to assume that the amount of reagent bound was proportional to the amount applied, which seems reasonable since only a small fraction of the total groups of any one type is modified when the activity is reduced to 10%. The linear course of such a plot with the line passing through the origin is usually taken as evidence of a single-hit reaction (Lea, '47), i.e., the reaction of one structure results in the loss of function for the whole unit. However, this applies to systems in which the effect of the reaction on the individual units can be evaluated directly, e.g., the colony-forming capacity of bacteria or plaque formation of a virus. In pepsin, the case is equivocal because there is no assay for individual enzyme molecules — only an average value of a large number can be obtained. For this reason, it is not possible to distinguish between the case in which the molecules

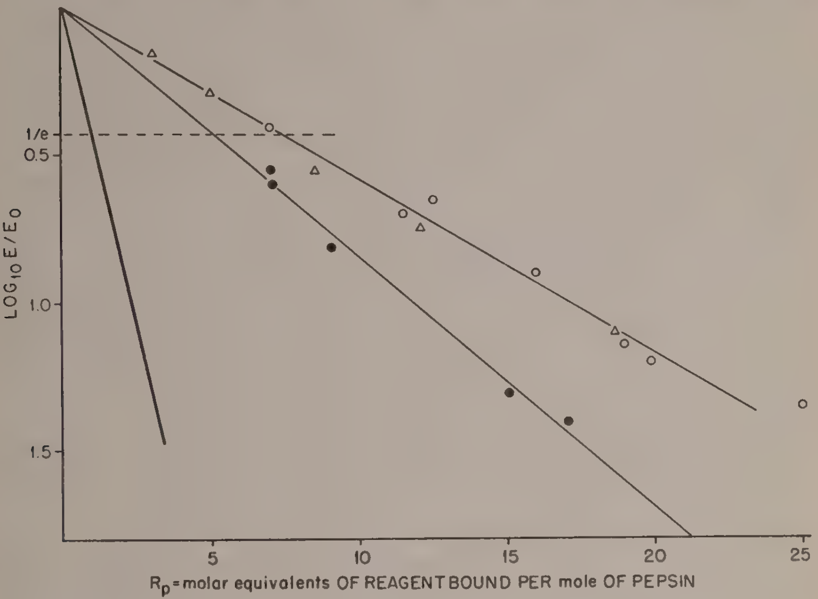

Fig. 1 Inactivation of pepsin. Heavy line is the theoretical curve for one reagent molecule inactivating one enzyme molecule.

Reagent		Slope × 2.3	*R* at 1/*e*
Sulfur mustard	(●)	0.20	5
Ketene	(△)	0.13	7.5
Iodine	(○)	0.13	7.5

are partially active from a mixture of inactive and fully active molecules. Therefore, from the present data it cannot be decided whether pepsin has one active site or several per molecule, but it is clear from the linear course of the results in figure 1 that the modification of only one structure is sufficient to destroy the catalytic activity of one site.

Despite this dilemma, some useful information can be obtained from a further examination of the theory. In its simplest form, the dosage reaction follows an exponential equation $E/E_0 = e^{-D}$, or $\ln E/E_0 = -D$, where E/E_0 is the fraction of enzyme activity remaining and D is the dose of inactivating agent. When D is 1, E/E_0 is 0.37, or when the activity is reduced to 37% of its original value, an average of one inactivating dose has been absorbed. *This is a maximal value, for it includes the reagent residues absorbed by structures having no relation to the active site.* In the present instance, this information can be used to obtain the maximum number of active sites on a pepsin molecule. The number of peptic carboxyl groups esterified by mustard, which reduces its enzymic activity to 37%, is five per molecule. As stated, a decision cannot now be made as to whether there are five active carboxyl groups having equal enzymic action or one active and four inactive that react under these circumstances. When the same reasoning is applied, an upper limit of the number of tyrosine groups associated with peptic activity is seven to eight per molecule. Since these upper limits include some inactive groups, the actual number of active groups is less than the figure noted and might be just one of each per molecule.

Professor Kauzmann (Herriott, '54) pointed out a simple means of calculating the relative rate of reaction of the particular protein groups associated with the active site as compared to that of the similar groups having essentially no close association with the activity. This calculation showed that the carboxyl groups associated with the peptic activity are nearly seven times as reactive with mustard gas as the other type.

To resolve the problem of the number of active sites of this enzyme, it will be necessary to use a very different approach. A dipeptide inhibitor of pepsin would permit a determination of its molar combining ratio and from that the number of active sites.

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KINETIC EFFECTS OF THE IONIZATION OF GROUPS IN THE ENZYME MOLECULE[1]

ROBERT A. ALBERTY
Chemistry Department, University of Wisconsin, Madison

FIVE FIGURES

HISTORICAL INTRODUCTION

The investigation of the effect of *p*H on enzymic reactions offers a means of studying the ionizable groups which are closely connected with the catalytic activity. A number of enzymic reactions have been studied in this way, and in most instances rather simple equations have been found to represent the data. The marked dependence of the enzymic activity on the ionization of one or two groups suggests that these groups may actually be involved in the exchanges of protons between enzyme and substrate. In this review, it is the objective to survey past accomplishments in this field and to discuss its present status from a unified point of view where other aspects of the physical chemistry of proteins are taken into account.

In 1911, Michaelis and Davidsohn advanced the theory that the dependence of enzymic activity on *p*H is a consequence of the amphoteric properties of proteins. On the basis of the very simple theory of protein ionization which prevailed at that time, they reasoned from the bell-shaped plots of activity versus *p*H that it was the isoelectric form of the enzyme which was catalytically active. The effect of *p*H on the invertase reaction was determined, and it was shown that the

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[1] This research has been supported by the National Science Foundation, by the Research Committee of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation, and by a Grant-In-Aid from the du Pont Company.

activity curve could be fitted quite well by an equation which allowed for one ionization with an acid dissociation constant of 2×10^{-7} and another with a base dissociation constant of 10^{-12}. This was even before the historic paper by Michaelis and Menten ('13). In maltase, Michaelis and Rona ('13) found that the anionic form rather than the isoelectric form was active; and in diastase, Michaelis and Pechstein ('14) showed that the rates were different in buffers of the same *p*H which contained salts having different anions but that the rates were the same if the salts had the same anion and different cations. In 1914, Michaelis discussed the ionization of enzymes and derived a number of simple equations. In the same year, van Slyke and Zacharias ('14) systematically investigated the *p*H dependence of the urease reaction and developed a theory to explain these results.

In 1920, Michaelis and Rothstein altered the theory of Michaelis and Davidsohn by attributing the ionization constant which had been determined for invertase to the enzyme-substrate complex rather than to the free enzyme. Willstäter and Kuhn ('23) suggested that if this were true the alkaline branch of the curve should shift with substrate concentration, and, since no shift was observed, they criticized the Michaelis theory. The situation was clarified by von Euler *et al.* ('24), who explained that this is just what would be expected if the free enzyme and the enzyme-substrate complex had the same ionization constant. The theory of these effects was further developed by Myrbäck ('26).

In 1930, Haldane reviewed the effect of *pH* on enzyme kinetics and pointed out that, in many cases, it was not possible to correlate activity with the isoelectric point and that it was more reasonable to assume that the activity depended on the ionization of certain groups in the enzyme molecule.

It is not possible to survey all the studies which have been made on the effects of *p*H on enzymic reactions. Of course very few of these studies are suitable as a basis for quantitative calculations, for reasons discussed later. Dixon ('53) has discussed the data available on the reactions catalyzed by urease, phosphatase, arginase, saccharase, fumarase, cholinesterase, and xanthine oxidase; and Laidler ('55c) has discussed the data for pepsin, chymotrypsin, cholinesterase, acetylcholinesterase, saccharase, histidase, acid phosphatase, urease, fumarase, and xanthine oxidase. Many of the experimental studies were quite preliminary, and more detailed results will be required to verify these interpretations. Special mention will be made of some of the studies in which the theory of *p*H effects was discussed. In 1944, Walker and Schmidt discussed the *p*H dependence of the kinetics of histidase and reported a *pK* value of 7.4 for the enzyme-substrate complex. The effect of *p*H on the arginase reaction was studied by Greenberg and Mohammed ('45), who concluded that the enzyme contained a group with $pK = 7.0$. The effect of *p*H on the luminescence intensity of *Photobacterium phosphoreum* has been investigated by Johnson and coworkers ('45). They found that the plot of luminescence intensity versus *p*H yields a symmetrical bell-shaped curve.

Myrbäck and coworkers have discussed the effect of *p*H on the reactions catalyzed by urease (Myrbäck, '47), amylase (Myrbäck, '51; Myrbäck and Björklund, '52; Myrbäck and Persson, '52), and saccharose (Myrbäck and Björklund, '52; Myrbäck and Persson, '52). In barley β-amylase, they found the alkaline branch of the curve to have the form expected for the ionization of a single group whose ionization constant (10^{-8}) is the same in the free enzyme and in the enzyme-substrate complex. The acid branch could be fitted quite well by allowing for three ionizable groups having the same ionization constant (5×10^{-4}).

Wilson and Bergmann ('50a, b) have investigated the effect of *p*H on the rate of hydrolysis of acetylcholine by acetylcholinesterase and found that the results can be interpreted in terms of the donation and acceptance of protons by the active site of the protein. They point out that the *pK* values which they obtain, 7.2 and 9.3 at 0.15 ionic strength, are suggestive of imidazolium and amino groups, respectively. Hearon ('52) has given a generalized steady-state treatment which automatically gives *p*H dependence.

In 1953, several important theoretical papers appeared in this field. Waley ('53) derived rate equations which allowed for the ionization of two groups in the enzyme and in the enzyme-substrate complex. Dixon ('53b) discussed the effect of *p*H on the affinities of enzymes for substrates and inhibitors and derived equations which were applied by Massey ('53b) to fumarase data. Dixon devised rules for making calculations of ionization constants from plots of the logarithm of the Michaelis constant or inhibition constant versus *p*H. Kistiakowsky and Shaw ('53) discussed the effect of *p*H on the action of urease in the range 5.5–7.0 in terms of the interacting site mechanism including inhibition by protons. Botts and Morales ('53) treated the effect of a single modifier, which might be a proton, by means of a complete steady-state treatment and discussed the conditions under which simple relations are obtained from the more general rate equation.

Friedenwald and Maengwyn-Davies ('54) have derived a number of rate equations allowing for various effects of *p*H by use of the rapid equilibrium assumption, and Laidler ('55a) has derived the rate equations for a quite general mechanism by use of the steady-state assumption and has discussed the conditions under which simpler forms are obtained.

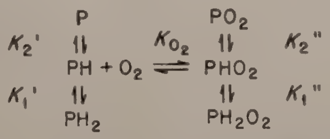
Since 1953 considerable research has been done on the effect of *p*H on the fumarase reaction, and the results will be discussed in some detail in this article.

IONIZATION OF HEME-LINKED GROUPS

Since the combination of hemoglobin with oxygen is analogous to the first step of an enzymic reaction, it is profitable to discuss the ionization of the heme-linked groups of hemoglobin before proceeding to enzymic reactions. The affinity of hemoglobin for oxygen varies with the *p*H, a necessary consequence of this being that protons are either displaced or used up when oxygen combines with hemoglobin. Above ~*p*H

6.4, the solution becomes more acid as the combination with oxygen proceeds, and at lower *p*H values it becomes more alkaline. A physiologically important result of this effect is that, in the lungs, carbon dioxide is displaced from bicarbonate. The average number of protons displaced per molecule of oxygen was determined by German and Wyman ('37) by titrating hemoglobin in the absence of oxygen and after saturation with oxygen. A plot of the difference between these titration curves versus *p*H is referred to as a "differential titration" curve. For hemoglobin, the differential titration curve and the variation of oxygen affinity with *p*H may be represented quantitatively by consideration of two groups which are the same for each heme, one of which is rendered stronger, and the other weaker, by oxygenation. The values of the ionization constants and their temperature coefficients strongly suggest that they are imidazole groups. Coryell and Pauling ('40) have shown that the acid-strengthening effect on one of the groups can be explained in terms of the change in bonding of the iron.

Whereas Wyman ('48) assumed for purposes of calculation that the two groups are independent, it has been shown (Alberty, '55) that values of the ionization constants may be obtained from equations derived without this restrictive assumption. If no assumption regarding the independence of the two ionizable groups is made, the active site of hemoblogin may be considered to be a dibasic acid and the equilibria involving molecular oxygen and protons may be represented by



$$\begin{tikzcd}
P \arrow[d, shift left] \arrow[d, phantom, "K_2'" left=6pt] & & PO_2 \arrow[d, shift left] \arrow[d, phantom, "K_2''" right=6pt] \\
PH + O_2 \arrow[u, shift left] \arrow[d, shift left] \arrow[rr, shift left, "K_{O_2}"] \arrow[d, phantom, "K_1'" left=6pt] & & PHO_2 \arrow[u, shift left] \arrow[ll, shift left] \arrow[d, shift left] \arrow[d, phantom, "K_1''" right=6pt] \\
PH_2 \arrow[u, shift left] & & PH_2O_2 \arrow[u, shift left]
\end{tikzcd} \tag{1}$$

where P stands for the part of hemoglobin with which one oxygen molecule combines and $K_{O_2} = p_{O_2}[PH]/[PHO_2]$. Arrows could also be written for the combination of oxygen with P or PH_2, but this is immaterial for the interpretation of equilibrium data since the equilibrium constants for these additional reactions are not independent of those for the other reactions. The acid dissociation constants are of the usual type for a dibasic acid, i.e.,

$$K_1' = [\mathrm{H}^+][\mathrm{PH}]/[\mathrm{PH}_2],\quad K_2' = [\mathrm{H}^+][\mathrm{P}]/[\mathrm{PH}],\ \text{etc.}$$

It may readily be shown that the variation with pH of the oxygen pressure required to half-saturate hemoglobin is given by

$$p_{½} = K_{\mathrm{O}_2}\,\frac{1 + [\mathrm{H}^+]/K_1' + K_2'/[\mathrm{H}^+]}{1 + [\mathrm{H}^+]/K_1'' + K_2''/[\mathrm{H}^+]} \qquad (2)$$

and that the number, $\Delta\,\overline{X}$, of equivalents of acid produced per heme upon oxygenation is given by:

$$\Delta\overline{X} = \frac{1 + 2[\mathrm{H}^+]/K_1'}{1 + [\mathrm{H}^+]/K_1' + K_2'/[\mathrm{H}^+]} - \frac{1 + 2[\mathrm{H}^+]/K_1''}{1 + [\mathrm{H}^+]/K_1'' + K_2''/[\mathrm{H}^+]} \qquad (3)$$

These equations are different from those obtained by Wyman and coworkers on the basis of the assumption of independence of the two acid groups, but the functional dependence on hydrogen ion concentration is the same; equations (2) and (3) can therefore represent the same theoretical curves as those calculated by Wyman.

The values of the ionization constants defined in equation (1), which yield the same theoretical curves as those given by Wyman, are summarized in table 1 as pK values (—log K). Once the values of K_1', K_2', K_1'', and K_2'' have been obtained, it is possible to calculate the microscopic constants [see equation (26)] on the basis of any of the following special assumptions: (a) the two groups are independent, (b) the two groups are identical but not independent, or (c) the intermediate ionized form PH is a particular isomer. The pK values calculated on assumption (a) are those of Wyman. The pK values obtained on the basis of assumption (c) are the same as the pK values calculated without making any assumption. For reasons to be discussed later, an electrostatic correction should be applied in the calculation of the ionization constants of the heme-linked groups, since the apparent ionization constants defined in equation (1) are expected to vary with the net charge on the protein molecule.

Theorell and coworkers (Theorell and Åkesson, '41; Theorell, '43) have carried out differential titration experiments with other heme proteins, including cytochrome c and ferroperoxidase. Spectroscopic shifts for peroxidase were observed at pH 4 and 5 by Theorell and Paul ('44), but in an attempt to find kinetic evidence for these groups, Chance ('52b) identified these changes as the first two steps in the splitting of heme from the protein.

TABLE 1

pK values for heme-linked groups in horse hemoglobin

(0.16 ionic strength at 25°C.)

		HEMOGLOBIN	OXYGENATED HEMOGLOBIN
pK_1		5.25	5.70
pK_2		7.93	6.73
Assumption (a):	pK_1	5.25	5.75
	pK_2	7.93	6.68
Assumption (b):	pK_1	5.55	6.00
	pK_2	7.63	6.43

The pH independence of the kinetic constants of catalase is quite striking. Chance ('52a) found no significant effect on the velocity of combination of catalase and methyl hydrogen peroxide in the range pH 2.2–12.0; the changes in reaction velocity beyond this range were attributable to the denaturation of catalase. Also, no effect of pH on the reaction of the catalase-hydrogen peroxide complex with ethanol was found in the range pH 4.2–12.0.

EFFECT OF pH ON THE CATALYSIS BY FUMARASE

Fumarase has proved to be a very suitable enzyme for a detailed study of the effect of pH. This enzyme catalyzes the reaction

$$F + H_2O \rightleftharpoons M \tag{4}$$

where the symbols, F, fumarate, and M, L-malate, include the various ionized forms of the substrates. At substrate concentrations below the range where substrate activation or inhibition occurs, the Michaelis-Menten equation is obeyed by both the forward and reverse reactions.

$$v = \frac{V_F}{1 + K_F/[F]} \text{ and } v = \frac{V_M}{1 + K_M/[M]} \qquad (5)$$

where v is the steady-state velocity at the initial substrate concentration. The maximum initial velocities V_F and V_M are directly proportional to the enzyme concentration whereas the Michaelis constants K_F and K_M are independent of enzyme concentration. Since the initial velocities of both the forward and reverse reactions may be determined, it is possible, as will be seen later, to check certain conclusions concerning the ionization of the enzyme.

In determining Michaelis constants and maximum velocities it is important to obtain the steady-state velocity which corresponds to the initial substrate concentration. The greatest difficulty is encountered at the lowest substrate concentrations, where the reaction is nearly first order. If, as in the reverse of reaction (4), only 20% of a substrate reacts before equilibrium is reached, it can be shown that the reaction is zero order within ± 5% for almost as long as it is first order within this precision. Thus it is convenient to determine the rate from a zero-order plot of the data for the initial period of the reaction. For the fumarase reaction, integration of the differential equation for first-order conditions shows that the extent of reaction must not be allowed to exceed 7.8% when fumarate is the substrate if it is desired to obtain the initial velocity with an error of less than 5% by a zero-order calculation. When L-malate is the substrate a similar calculation shows that, because of the unfavorable equilibrium, only the first 1.7% of the reaction may be used. Greater extents of reaction may be used at concentrations in the neighborhood of the Michaelis constant or above.

The apparent equilibrium constant for the fumarase reaction, when written in terms of the total molar concentrations of fumarate and L-malate, is

$$K_{app} = \frac{[M]_{eq}}{[F]_{eq}} = K_{eq}\,\frac{1 + [H^+]/K_{HM}}{1 + [H^+]/K_{HF}}. \qquad (6)$$

This apparent equilibrium constant will vary with the pH according to equation (6) if only F^{--}, HF^{-}, M^{--}, and HM^{-} are present at appreciable concentrations (Bock and Alberty, '53). The secondary ionization constants for malate and fumarate are represented by K_{HM} and K_{HF}. The constant K_{eq} depends on the temperature and salt concentration, and has a value of 4.4 at 25°C. and an ionic strength of 0.01.

The first studies of the effect of pH on the kinetics of the fumarase reaction over an extended range were made by Massey ('53a), who investigated the effects of various anions and found that rather symmetrical bell-shaped plots of initial reaction velocity versus pH were obtained. Massey observed that a number of polyvalent anions such as phosphate, citrate, and sulfate altered predominantly the alkaline side of the activity-pH curves, whereas inhibiting monovalent anions such as chloride, bromide, and thiocyanate altered the acidic side. He made the important suggestion that the effects of these anions, both activating and inhibiting, could be explained largely in terms of their effect on the ionization constants of acidic and basic groups in the neighborhood of the active center. Later Massey ('53b), using phosphate buffers, also determined the values of the Michaelis constants for fumarate and L-malate and the competitive inhibition constants for a series of compounds over a range of pH. These data were interpreted with the aid of the theoretical equations of Dixon ('53). Complications to be discussed later are introduced by the use of phosphate buffers in studying fumarase.

Massey and Alberty ('54) showed that the plots of maximum initial velocity versus pH for both the forward and reverse reactions were symmetrical, bell-shaped curves which could be fitted by a simple equation yielding the ionization constants of the enzyme-substrate complexes K_{aEF}, K_{bEF}, K_{aEM}, and K_{bEM}. The values of these constants are essentially independent of the concentration of phosphate buffer over the range 5–133 mM. In continuing this research, Alberty *et al.*

('54) found that no simple interpretation could be given for the pH variation of the Michaelis constants in phosphate buffer.

The Haldane equation. In spite of all the complications encountered with phosphate buffers, it has been found (Bock and Alberty, '53; Alberty *et al.,* '54) that the maximum initial velocities and Michaelis constants for the forward and reverse fumarase reactions are related to the equilibrium constant for the over-all reaction by an equation first derived by Haldane ('30).

$$K_{\mathrm{app}} = \frac{V_{\mathrm{F}} K_{\mathrm{M}}}{V_{\mathrm{M}} K_{\mathrm{F}}} \tag{7}$$

Equation (7) is obeyed over a wide range of conditions, provided that the kinetic constants are determined in the low substrate concentration range where there is no activation or inhibition by substrate. More complicated relations of this type are obtained for reactions involving coenzymes or other reactants and the various equations offer a means of testing the suitability of particular mechanisms (Alberty, '53).

Equation (7) may be derived (Alberty, '54) from all sorts of mechanisms which yield the Michaelis-Menten equation for initial rates. When the Michaelis-Menten equation is obeyed, the velocity at very low substrate concentrations is proportional to the substrate concentration. Under these conditions, the first-order rate constant for the forward reaction is $V_{\mathrm{F}}/K_{\mathrm{F}}$ and for the reverse reaction, $V_{\mathrm{M}}/K_{\mathrm{M}}$. Thus the Haldane equation may be considered to be a consequence of the equality of the ratio of the specific rate constants for the forward and reverse reactions to the equilibrium constant for a simple reversible first-order reaction.

Equation (7) holds even for the apparent maximum velocities and Michaelis constants obtained by use of a reaction medium containing an inhibitor or activator, provided that the rate data obey the Michaelis-Menten equation and substrate activation or inhibition is not involved. If substrate activation or inhibition occurs, the kinetic properties of the enzyme are different when the forward and reverse reactions are studied, and the Haldane equation would not be expected to be obeyed.

It is a corollary of equation (7) that over a range of pH, buffer concentration, or inhibitor concentration in which K_{app} is a constant, V_F/K_F must vary in the same way as V_M/K_M. Such relations are a constant reminder that an explanation for an effect on the forward reaction must also be applicable in interpreting the results for the reverse reaction. Thus it is advantageous to be able to study the reverse reaction in order to see whether this is true.

Experiments with buffers containing monovalent anions. It is found that, although phosphate buffers both activate and inhibit fumarase, substitution of potassium and ammonium ions for sodium ions is without effect on the kinetics. It is to be expected that the effect of bound $H_2PO_4^-$ ions will be different from bound HPO_4^{--} ions so that the magnitudes of the activating and inhibiting effects change with the pH at constant phosphate concentration. A buffer utilizing an uncharged base or uncharged acid has the advantage over a phosphate buffer that the concentration of a single anion may be held constant over a wide range of pH.

The maximum initial velocities and Michaelis constants shown in figure 1 were obtained by Frieden and Alberty ('55) using tris-(hydroxymethyl)-aminomethane acetate buffers of 0.01 ionic strength. It will be noted that the pH optima for the forward and reverse reactions are quite different and that the Michaelis constants for the forward and reverse reactions vary in a different way with the pH. The lines through the experimental points have been calculated by use of equations presented in a later section. Because of the Haldane equation, only three of these theoretical lines are independent, and the theoretical curve for the Michaelis constant of fumarate has been drawn based on constants determined from the other three curves and the equilibrium constant for the over-all reaction. The theoretical curves have been fitted to the data in the *p*H range of ~5.5–8.0, and it is seen that there are pronounced deviations at higher *p*H values. Possible reasons for these deviations will be discussed later. In order to show the effect of substituting one monovalent salt for another, the variation with *p*H of the maximum initial velocities and Michaelis constants for L-malate in 0.005 *M* perchlorate and chloride solutions are shown in figure 2. Again, deviations from the theoretical curves are obtained at high *p*H values.

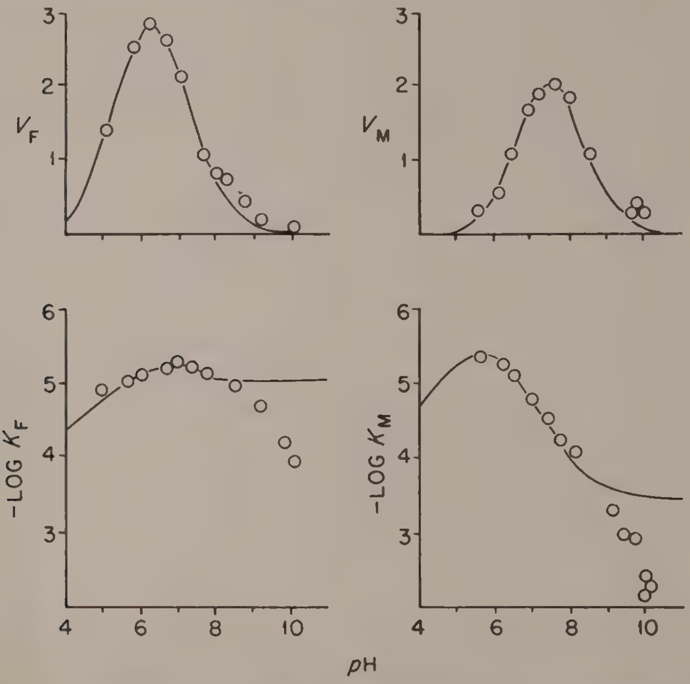


Fig. 1 Variation with *p*H of maximum initial velocities and Michaelis constants (in moles per liter) for the fumarase reaction in 0.01 *M* acetate buffers at 25°C. (Frieden and Alberty, '55). The experimental data are represented by points and the theoretical curves have been calculated with equations (12) and (13) with constants given in table 3.

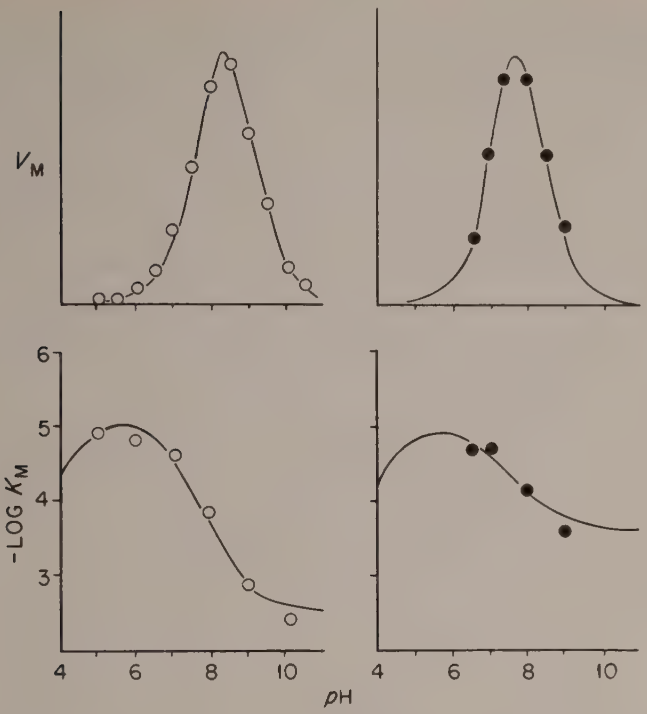


Fig. 2 Variation with *p*H of the maximum initial velocities and Michaelis constants (in moles per liter) for L-malate in 0.005 *M* perchlorate (open circles) and chloride (solid circles) solutions at 25°C. (McKinley McKee, personal communication). The experimental data are represented by points and the theoretical curves have been calculated with equations (12) and (13).

PROTEIN IONIZATIONS

Thermodynamic ionization constants. An enzyme molecule may be considered to be a polyvalent acid H_mP whose successive ionizations are represented by m thermodynamic ionization constants.

$$\begin{aligned} H_mP &\rightleftharpoons H_{m-1}P + H^+ \qquad & K_1 &= \frac{[H^+][H_{m-1}P]}{[H_mP]} \\ &\vdots & &\vdots \\ HP &\rightleftharpoons P + H^+ & K_m &= \frac{[H^+][P]}{[HP]} \end{aligned} \tag{8}$$

where the parentheses indicate activities. With the exception of H_mP and P, each of these symbols represents the sum of a large number of isomers. There are $m!/h![m-h]!$ isomers of $H_{m-h}P$ and a total of 2^m forms of the protein (Cohn and Edsall, '43). For example, there are 10^{29} isomers of a protein containing 100 ionizable groups that has 50 protons dissociated.

In principle, it would be possible to determine the m thermodynamic ionization constants for a protein but, in practice, this is impossible because m is such a large number and many of the groups ionize in the same pH range. Therefore, a different point of view (described in the next section) is adopted in the interpretation of protein titration curves.

Apparent ionization constants of groups in proteins. In order to reduce the number of constants required to express the titration curve, the ionizable groups of a protein are divided into a small number of classes, depending on their ionization constants. If all the groups in a class have the same tendency to ionize in the absence of electrostatic effects of charged groups in a molecule, their degree of ionization may be expressed in terms of a single constant. The ionization constants obtained in this way give a more direct representation of the tendency of the groups of the class to ionize than do the m thermodynamic ionization constants. However, unlike the thermodynamic constants, they are not absolutely constant, but change with the net charge on the protein molecule. It is not possible to take this effect into account in an exact way, and so it is necessary to adopt a model which is amenable to theoretical treatment. The model first suggested by Linderstrøm-Lang ('24) and further developed by Scatchard ('49) is a spherical molecule with the ionized groups distributed uniformly on the surface. It was shown that the ionization constant of a particular group may be expressed

$$K = K_0 \exp 2zw \tag{9}$$

where K_0 is the value the ionization constant would have if the protein were isoelectric, and z is the net charge on the protein molecule. The electrostatic factor w is defined so that $2RTwz$ is the electrostatic work which must be done to remove a univalent ion such as a hydrogen ion from the surface of the protein molecule to infinity. In this expression R is the ideal gas constant and T is the absolute temperature.

For the idealized model of a spherical protein molecule with the charges distributed uniformly on the surface, w is given by

$$w = \frac{Ne^2}{2\mathrm{DRT}} \left(\frac{1}{b} - \frac{k}{1 + ka} \right) \tag{10}$$

where N is Avogadro's number, e is the charge on the electron, D is the dielectric constant of water, b is the radius of the protein sphere, a is the radius of exclusion, and k is the constant occurring in the Debye-Hückel theory.

Equation (9) may also be considered to give an empirical definition of w. The values of w calculated from titration data by equation (9) have always turned out to be equal to or smaller than those calculated from the theoretical equation (10) (Tanford, '55). Since the theoretical calculation of w depends on the model used, it may be expected that better equations than (10) will be derived as the knowledge of protein structure improves.

EFFECTS OF IONIZATION ON KINETICS

The general variation with pH in the velocities of enzymic reactions, even in regions where the degree of ionization of the substrate is not changing, indicates that different ionized forms of the enzyme have different catalytic properties. If the catalytic activity was simply owing to the form of the enzyme having a certain number of hydrogens dissociated, e.g., all the isomers $\mathrm{H}_{m-h}\mathrm{P}$, then the thermodynamic constants defined in (8) would express the pH dependence of activity. However, this situation is very unlikely since it is not possible to imagine why the catalysis should depend simply on the total number of groups in the protein molecule which are ionized. It appears more likely that the catalysis is affected primarily by the ionization of groups in the enzymic site and its immediate vicinity.

For the purpose of considering rapidly reversible effects of ionization on catalytic activity, the groups can be divided arbitrarily into three classes:

I. Groups that must be in a particular ionized form in order for the catalytic reaction to occur at all. These groups may be said to have a total effect on the activity.
II. Groups in the neighborhood of the enzymic site that have a large but nontotal effect on the kinetics.
III. All the rest of the groups of the protein molecule.

Since the effect of pH on enzyme kinetics can frequently be represented by use of one to four ionization constants, many enzymic reactions may involve groups of class I. If the various complexes and ionized forms are in rapid equilibrium in solution, it is unnecessary to make further arbitrary divisions with respect to whether the substrate can combine with various ionized forms of the enzyme. This is taken care of by the magnitude of the equilibrium constant for the reaction with a particular ionized form of the protein and the various ionization constants of the enzyme and enzyme-substrate complexes.

Groups of class III are generally ignored in interpreting enzyme kinetic data, but they can be expected to exert an electrostatic effect, at the very least, on the groups at the catalytic site and the various rate processes. The nature of these electrostatic effects will be discussed after a consideration of groups of class I. The mathematical representation of the effect of groups of class II becomes quite complicated and will not be discussed. Mechanisms incorporating such effects have been discussed by Laidler ('55a).

Effects of groups in the enzymic site (Class I). In order to account for a pH optimum in the maximum initial velocity, it is necessary to postulate at least two ionizable groups. In the following mechanism, the enzymic site is represented as a dibasic acid EH_2.

$$\begin{tikzcd}
E \arrow[d, shift left] & & ES \arrow[d, shift left] & \\
EH + S \arrow[u, shift left, "K_{bE}"] \arrow[d, shift left] \arrow[rr, shift left, "k_1"] & & EHS \arrow[u, shift left, "K_{bES}"] \arrow[ll, shift left, "k_2"] \arrow[r, "k_3"] \arrow[d, shift left] & EH + P \\
EH_2 \arrow[u, shift left, "K_{aE}"] & & EH_2S \arrow[u, shift left, "K_{aES}"] &
\end{tikzcd} \qquad (11)$$

The acid dissociation constants K_a and K_b are of the usual type for a dibasic acid, i.e., $K_{a\mathrm{E}} = [\mathrm{H}^+][\mathrm{EA}]/[\mathrm{EH}_2]$, $K_{b\mathrm{E}} = [\mathrm{H}^+][\mathrm{E}]/[\mathrm{EH}]$, $K_{a\mathrm{ES}} = [\mathrm{H}^+][\mathrm{EHS}]/[\mathrm{EH}_2\mathrm{S}]$, and $K_{b\mathrm{ES}} = [\mathrm{H}^+][\mathrm{ES}]/[\mathrm{EHS}]$. As indicated by the subscripts, the ionization constants of the enzyme-substrate complex may be different from those for the free enzyme. The symbol EHS represents the ionized form of the enzyme-substrate complex which yields product but which loses its activity either upon addition or loss of a proton.

Under the usual condition that $[\mathrm{S}] >> [\mathrm{E}]_0$ where $[\mathrm{E}]_0 = [\mathrm{E}] + [\mathrm{EH}] + [\mathrm{EH}_2] + [\mathrm{ES}] + [\mathrm{EHS}] + [\mathrm{EH}_2\mathrm{S}]$, the steady-state treatment of this mechanism yields the Michaelis equation for the initial velocity with the *p*H dependence of the maximum initial velocity V_S and the Michaelis constant K_S given by

$$V_\mathrm{S} = \frac{k_3[\mathrm{E}]_0}{1 + [\mathrm{H}^+]/K_{a\mathrm{ES}} + K_{b\mathrm{ES}}/[\mathrm{H}^+]} \tag{12}$$

$$K_\mathrm{S} = \frac{(k_2 + k_3)}{k_1}\,\frac{1 + [\mathrm{H}^+]/K_{a\mathrm{E}} + K_{b\mathrm{E}}/[\mathrm{H}^+]}{1 + [\mathrm{H}^+]/K_{a\mathrm{ES}} + K_{b\mathrm{ES}}/[\mathrm{H}^+]}. \tag{13}$$

This is the same result as that obtained by Waley ('53), whereas Dixon ('53) has discussed an equation analogous to equation (13) which gives the *p*H dependence of the Michaelis constant. These are the equations which have been used to calculate the theoretical lines in figures 1 and 2 with the exception of an additional factor in equation (13) to allow for the ionization of the substrate.

If reactions such as $\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES}$ or $\mathrm{EH}_2 + \mathrm{S} \rightleftharpoons \mathrm{EH}_2\mathrm{S}$ occur more rapidly than $\mathrm{EH} + \mathrm{S} \rightleftharpoons \mathrm{EHS}$, a different steady-state rate equation will be obtained. The rate equations for such mechanisms have been discussed by Laidler ('55a), who finds that the expressions given in equations (12) and (13) are obtained only when the various equilibria are adjusted rapidly or the reaction $\mathrm{EH} + \mathrm{S} \rightleftharpoons \mathrm{EHS}$ is so much faster that it predominates. If reactions such as $\mathrm{EH}_2 + \mathrm{S} \rightleftharpoons \mathrm{EHS} + \mathrm{H}^+$ occur, still different rate expressions are obtained. If all the equilibria are adjusted rapidly it makes no difference, of course, where the arrows are placed in the mechanism provided that all the important complexes and ionized species are included; and if this is the case, equation (12) is unchanged and equation (13) is the same except that k_3 in the numerator disappears. Thus, although these equations have been calculated from a steady-state treatment, it is questionable if this is much better than calculation based on the assumption of rapid equilibrium.

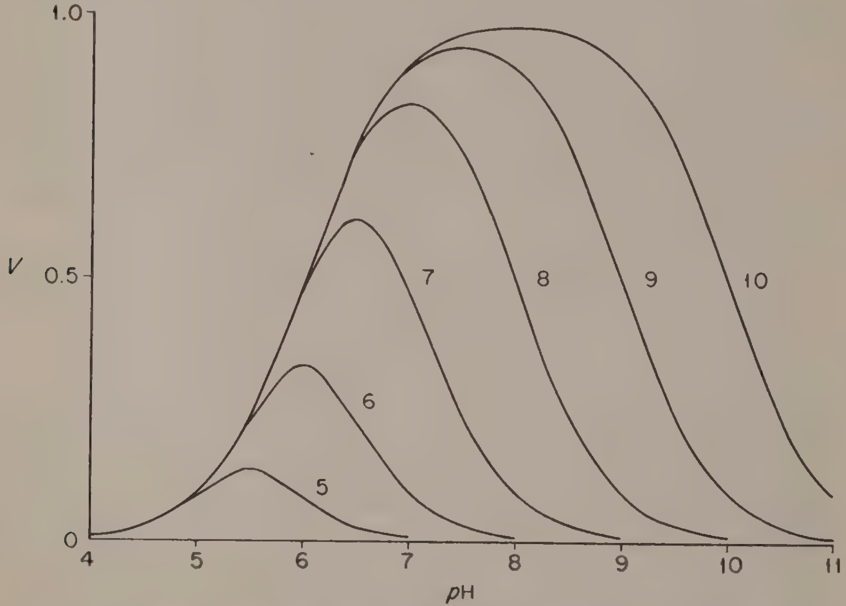


Fig. 3 Variation of V_S with pH according to equation (12) with $k_3[E]_0 = 1$, $pK_a = 6.00$ and for values of pK_b given on the graph (Alberty and Massey, '54).

Equation (12) represents the bell-shaped plot of V_S versus pH which is frequently obtained experimentally. A series of plots for $K_{a\mathrm{ES}} = 10^{-6}$ and a variety of values of $K_{b\mathrm{ES}}$ are given in figure 3 for $k_3[E]_0 = 1$. If the enzyme-substrate complex all existed in the ionized form represented by EHS, the velocity would be 1; but since there are three ionized forms of the enzyme-substrate complex, this velocity is not obtained unless $pK_{a\mathrm{ES}}$ and $pK_{b\mathrm{ES}}$ are well separated. The value of $K_{b\mathrm{ES}}$ will generally be smaller than $K_{a\mathrm{ES}}$, although this is not necessary. In the unusual case where the dissociation of the first proton causes an acid-strengthening effect, $K_{b\mathrm{ES}}$ will be greater than $K_{a\mathrm{ES}}$.

The pH dependence of competitive inhibition to be expected for the preceding mechanism may be obtained by adding the following equilibria.

$$\begin{tikzcd}
\mathrm{E} \arrow[d, shift left] & \mathrm{EI} \arrow[d, shift left] \\
\mathrm{EH} + \mathrm{I} \arrow[u, shift left, "K_{bE}"] \arrow[d, shift left] \arrow[r, shift left, "K_{EI}"] & \mathrm{EHI} \arrow[u, shift left, "K_{bEI}"'] \arrow[l, shift left] \arrow[d, shift left] \\
\mathrm{EH_2} \arrow[u, shift left, "K_{aE}"] & \mathrm{EH_2I} \arrow[u, shift left, "K_{aEI}"']
\end{tikzcd} \qquad (14)$$

The pH dependence of the competitive inhibition constant (Alberty, '54) is given by

$$K_{\mathrm{I}} = K_{\mathrm{EI}} \frac{1 + [\mathrm{H}^+]/K_{a\mathrm{E}} + K_{b\mathrm{E}}/[\mathrm{H}^+]}{1 + [\mathrm{H}^+]/K_{a\mathrm{EI}} + K_{b\mathrm{EI}}/[\mathrm{H}^+]} \qquad (15)$$

It is apparent from this equation that the magnitude of the competitive inhibition constant depends on both the affinity of the inhibitor for a particular ionized form of the enzyme, as represented by K_{EI}, and the effect of the inhibitor on the ionization constants of the groups of the enzymic site. Thus, for an enzymic reaction which follows a mechanism of this type, it would plainly be impossible to interpret correctly the effect of various structural features of inhibitors upon the value of K_{I}. However, by studies over a wide range of pH, it should be possible to determine the value of $K_{a\mathrm{EI}}$, $K_{b\mathrm{EI}}$ and K_{EI}. In this way, the effect of the inhibitor on the ionization constants of essential groups could be considered separately from the affinity of the inhibitor for a particular ionized form of the enzymic site.

Methods for calculating ionization constants. It is evident from figure 3 that if pK_b is greater than pK_a by 3 or more units, the pK values may be simply read off the curve at the pH's at which V_{S} has half its maximum value. However, if the pK values are closer together, a different method of calculation is necessary. It has been shown (Alberty and Massey, '54) that the values of $K_{a\mathrm{ES}}$ and $K_{b\mathrm{ES}}$ may be calculated conveniently by use of the equations

$$K_{a\mathrm{ES}} = [\mathrm{H}^+]_a + [\mathrm{H}^+]_b - 4\sqrt{[\mathrm{H}^+]_a[\mathrm{H}^+]_b} \tag{16}$$

$$K_{b\mathrm{ES}} = \frac{[\mathrm{H}^+]_a[\mathrm{H}^+]_b}{K_{a\mathrm{ES}}} \tag{17}$$

where $[\mathrm{H}^+]_a$ and $[\mathrm{H}^+]_b$ are the hydrogen ion concentrations on the acidic and basic sides, respectively, at which V_S is one-half that at the optimum pH. It should be pointed out that the closer $p\mathrm{H}_a$ and $p\mathrm{H}_b$ are together, the greater is the error in the calculated values of pK_a and pK_b that results from a given error in $p\mathrm{H}_a$ and $p\mathrm{H}_b$. This is illustrated by the data in table 2, which gives $p\mathrm{H}_a$ and $p\mathrm{H}_b$ for a series of pK_b values when pK_a is held constant at 6.00. The error given in the last column results if the experimental value of the pH at half-maximum velocity on the acidic side is 0.05 pH units too low and that at half-maximum velocity on the basic side is 0.05 too high. The ΔpH column gives the widths of the plots at half-maximum velocity and is a useful guide in indicating the relative magnitudes of pK_a and pK_b.

TABLE 2

Error in calculation of pK_a

($pK_a = 6.00$)

pK_b	pH_a	pH_b	ΔpH	ERROR IN pK_a
10	5.98	10.02	4.04	0.05
9	5.95	9.05	3.10	0.06
8	5.86	8.14	2.28	0.07
7	5.65	7.35	1.70	0.10
6	5.32	6.68	1.36	0.20
5	4.89	6.11	1.22	0.40

In calculating $pK_{a\mathrm{E}}$ and $pK_{b\mathrm{E}}$, it is convenient to take advantage of the fact that the pH dependence of the denominators of V_S and K_S are the same so that the values of $K_{a\mathrm{E}}$ and $K_{b\mathrm{E}}$ may be evaluated from a plot of $V_\mathrm{S}/K_\mathrm{S}$ versus pH.

$$\frac{V_\mathrm{S}}{K_\mathrm{S}} = \frac{k_1k_3[\mathrm{E}]_0/(k_2+k_3)}{1 + [\mathrm{H}^+]/K_{a\mathrm{E}} + K_{b\mathrm{E}}/[\mathrm{H}^+]} \tag{18}$$

The ratio V_S/K_S is simply the reciprocal of the slope of a plot of $1/v$ versus $1/[S]$. Plots of this type for the forward and reverse reactions are given in figure 4. To obtain simple, bell-shaped curves, it is necessary to plot $V_F(1 + [H^+]/K_{HF})/4.4\,K_F$ and $V_M(1 + [H^+]/K_{HM})/K_M$ versus *p*H, as is apparent from equations (6) and (7).

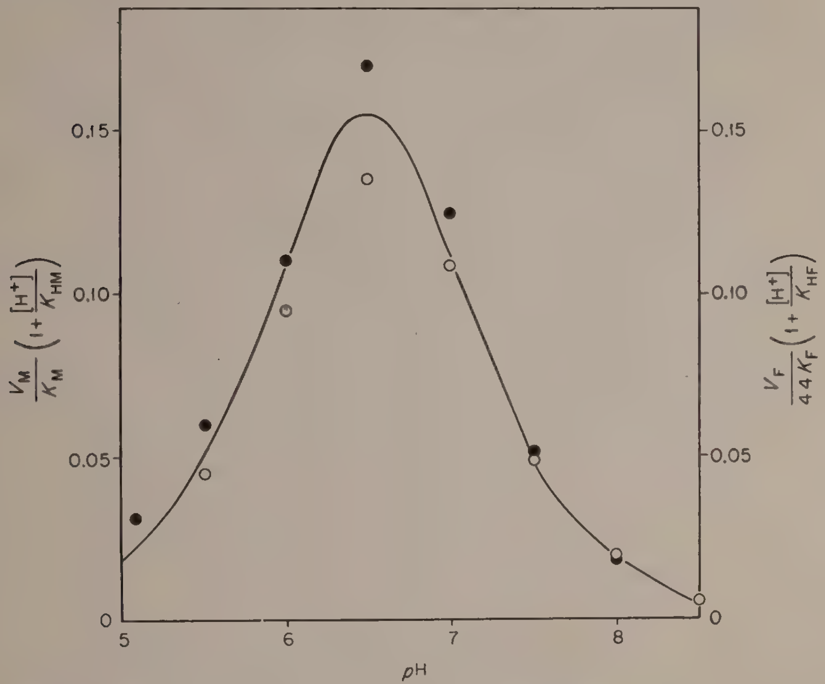


Fig. 4 Plot of $V_F(1 + [H^+]/K_{HF})/4.4\,K_F$ (solid circles) and $V_M(1 + [H^+]/K_{HM})/K_M$ (open circles) versus *p*H for 0.01 *M* acetate buffers at 25°C. The theoretical curve has been calculated by equation (18) (Frieden and Alberty, '55).

The values of K_{aEI} and K_{bEI} for a competitive inhibitor may be calculated from a plot of K_IV_S/K_S versus *p*H.

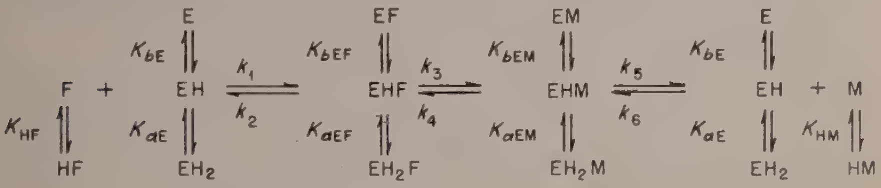
$$\frac{K_IV_S}{K_S} = \frac{k_1k_3[E]_0K_{EI}/(k_2 + k_3)}{1 + [H^+]/K_{aEI} + K_{bEI}/[H^+]} \qquad (19)$$

Dixon ('53) has given rules for the calculation of the various ionization constants from plots of $-\log K_S$ and $-\log K_I$ versus *p*H. For example, if such a plot contains two linear regions, one horizontal and the other with a slope of unity, with a curved region joining them, the pK value may be obtained from the intersection of the two linear sections. At this point, the curved line through the experimental points should lie above or below the intersection by 0.3 unit. The disadvantage of this method is that, if the various pK values are closely spaced, it may be impossible to identify the various changes in slope. Friedenwald and Maengwyn-Davies ('54) have suggested evaluating the constants from a plot of $1/V$ versus $([H^+]_0/[H^+] + [H^+]/[H^+]_0)$ where $[H^+]_0$ is the hydrogen ion concentration at the optimum pH. This method has the disadvantage of requiring the optimum pH to be read from the graph before the linear plot is made.

Effect of the binding of buffer ions. It is important to remember that the ionization constants, rate constants, and equilibrium constants in equations (12), (13), and (15) are not absolutely constant but vary with (a) specific effects owing to the binding of buffer ions, (b) nonspecific buffer effects (ionic strength), and (c) the net charge on the protein molecule. If components of the buffer exert specific effects on the rates, buffer ion binding may be included in the mechanism. For fumarase, the Michaelis constants, maximum velocities, and competitive inhibition constants are very dependent on the concentration of phosphate buffer. The increase in V_S with phosphate concentration indicates an activation resulting from the binding of phosphate at sites other than the enzymic site. The additional fact that K_S and K_I are apparently directly proportional to phosphate concentration indicates an inhibition resulting from combination of phosphate ions with the enzyme in such a way as to block the enzymic site. Even mechanisms including equilibria such as $E + B \rightleftharpoons BE$ lead to equations of the same form as (12), (13), and (15), *at constant buffer ion concentration.* However, K_{aE}, K_{bE}, K_{aES}, K_{bES}, etc., now become functions of buffer concentration (Alberty, '54). Even in the absence of such specific effects, these apparent ionization constants and binding constants for buffer ions and substrate ions will be expected to vary with the ionic strength of the buffer medium.

Buffers such as phosphate present an additional complication, since the ratio of the concentrations of monovalent and divalent ions changes with the pH. If these two ions have different effects on the enzyme, further equilibria must be added to the mechanism, and as a result the equation derived for V_S cannot be arranged in a symmetrical form. Buffers of the uncharged-acid or uncharged-base types have the advantage over phosphate that the concentration of the ionized form of the buffer may be held constant over a wide range of pH.

Application to fumarase. Equations (12) and (13) are found to represent the kinetic data obtained with 0.01 *M* tris-(hydroxymethyl)-aminomethane acetate buffers quite satisfactorily in the range pH 5.5–8.5 as illustrated in figure 1 (Frieden and Alberty, '55). The following mechanism, which allows for both the forward and reverse reactions, is the simplest mechanism which can represent all the facts at constant buffer concentration.



(20)

In this mechanism, F and M represent the doubly charged anions of fumarate and L-malate, which are the predominant forms of these substances existing above pH 5.5, and HF and HM represent the singly charged anions.

When both the forward and reverse reactions are included, the complete steady-state treatment shows that the ionization constants of the enzyme-substrate complexes determined from equation (12) are not simply interpretable as ionization constants of the essential groups of the active site. The change in interpretation is most conveniently represented by replacing K_{aES} and K_{bES} in equations (12) and (13) by apparent ionization constants $K'_{a\mathrm{EF}}$, $K'_{b\mathrm{EF}}$, $K'_{a\mathrm{EM}}$, and $K'_{b\mathrm{EM}}$. The relations between the apparent ionization constants obtained from the experimental data and the ionization constants defined in mechanism (20) are

$$K'_{a\mathrm{EF}} = \frac{(k_3 + k_4 + k_5)}{(k_4 + k_5)/K_{a\mathrm{EF}} + k_3/K_{a\mathrm{EM}}}, \tag{21}$$

$$K'_{b\mathrm{EF}} = \frac{(k_4 + k_5)K_{b\mathrm{EF}} + k_3 K_{b\mathrm{EM}}}{(k_3 + k_4 + k_5)}, \tag{22}$$

$$K'_{a\mathrm{EM}} = \frac{(k_2 + k_3 + k_4)}{(k_2 + k_3)/K_{a\mathrm{EM}} + k_4/K_{a\mathrm{EF}}}, \tag{23}$$

$$K'_{b\mathrm{EM}} = \frac{(k_2 + k_3)K_{b\mathrm{EM}} + k_4 K_{b\mathrm{EF}}}{(k_2 + k_3 + k_4)}. \tag{24}$$

To take a specific example, equation (21) shows that the experimentally determined value of $K'_{a\mathrm{EF}}$ may be equal to $K_{a\mathrm{EF}}$ or $K_{a\mathrm{EM}}$ or may fall between these values, depending on the relative values of k_3 and $(k_4 + k_5)$. If k_5 is the rate constant for the rate-determining step of the forward reaction at infinite fumarate concentration and k_2 is the rate constant for the rate-determining step of the reverse reaction at infinite L-malate concentration, then it follows from equations (21) to (24) that the *p*H dependence of the maximum velocity will be the same for the forward and reverse reactions. It is clear from figure 1 that this is not the case for fumarase.

On the other hand, if the steps with rate constants k_3 and k_4 are rate determining, the primed ionization constants become identical with the unprimed quantities defined by the mechanism in equation (20). The values of the ionization constants calculated on this assumption are summarized in table 3 as *pK* values. It is important to note that the full steady-state treatment shows that $K_{a\mathrm{E}}$ and $K_{b\mathrm{E}}$ can be calculated in the usual way without such assumption being made.

As a result of the change in *pK* values upon reaction, the complexes of fumarase with fumarate or L-malate at a particular *p*H value do not contain the same number of protons as that associated with the enzyme and substrate separately. Thus acid will be produced or consumed in the various steps of the reaction just as in the oxygenation of hemoglobin.

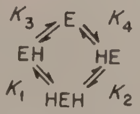
Although it might be argued that the term "enzyme-substrate complex" should not be used here, there seems to be no disadvantage in use of this term provided that it is remembered that protons may be lost or gained in the reaction. If there is a change in the number of water molecules associated with the reactants and products, no kinetic evidence at all can be obtained from studies in dilute aqueous solutions since the order of the reaction with respect to water cannot be determined.

TABLE 3

pK values of groups in fumarase and fumarase-substrate complexes in 0.01 M acetate buffers

	E (fig. 4)	EF (fig. 1)	EM (fig. 1)
pK_a	6.2	5.3	6.6
pK_b	6.8	7.3	8.4

The ionization constants in table 3 are not the intrinsic ionization constants for individual groups but rather are defined in the usual way for a dibasic acid. The relation between these constants and the intrinsic constants for individual groups is readily derived on the basis of the following reaction scheme (Adams, '16).



(25)

The usual ionization constants K_a and K_b for a dibasic acid are given by

$$K_a = K_1 + K_2$$
$$K_b = (1/K_3 + 1/K_4)^{-1}. \qquad (26)$$

A special case of interest is that in which $K_1 = K_2 = K_3 = K_4$ so that $K_a = 4K_b$. It will be noted that this is exactly the ratio of the constants $K_{a\mathrm{E}}$ and $K_{b\mathrm{E}}$ for fumarase. Thus it is possible that the two groups in the enzymic site have identical intrinsic constants under these conditions ($pK = 6.5$).

The fact that the pK values for the essential groups of fumarase fall in this range is very suggestive that they are imidazole groups of histidine. Tanford and Wagner ('54) give the following values for the pK values of imidazole groups in the isoelectric protein at zero ionic strength: ovalbumin, 6.7; insulin, 6.4; serum albumin, 6.3; β-lactoglobulin, 6.8; and lysozyme, 6.5–7.0. The isoelectric point of fumarase in 0.21 ionic strength phosphate buffer has been found to be between 5.0 and 5.4 (Massey, '52) and so the isoelectric point in 0.01 ionic strength acetate buffer is very likely in the range pH 6–7. Since the electrostatic correction on a pK value in this range would presumably be quite small, a direct comparison can be made between $pK = 6.5$ for fumarase and the listed pK values for other proteins. Since fumarase appears to be a simple protein (Frieden *et al.*, '54), the magnitude of the pK values is the only hint as to the nature of the groups in the active site.

The deviations from the theoretical curves found above pH 8 may indicate further ionizable groups in the enzymic site, but such deviations may also result from the electrostatic effects of the other charged groups in the protein molecule.

Effect of the net electrical charge of the enzyme molecule. Enzymic reactions are usually discussed without regard to that part of the protein molecule outside the enzymic site. However, three types of effects are to be expected purely on the basis of electrostatic interactions between the enzymic site and the charged groups in the remainder of the protein molecule. These effects are: (a) displacement of the pK values of ionizable groups in the enzymic site, (b) shifts in the equilibrium constants for the binding of substrate and buffer, and (c) alteration of the rate constants of steps in the enzymic reaction. In the study of these effects, there is an opportunity to apply knowledge of the physical chemistry of proteins. The following discussion will indicate the need for new experimental and theoretical studies.

In order to provide a mathematical basis for the discussion of the first effect, a larger number of ionizations may be included in the mechanism. The resulting rate equation will perforce involve a larger number of constants and higher powers of the hydrogen ion concentration. Since there is a limit to the number of constants which can be evaluated from kinetic data, it is necessary to find ways of simplifying such a general treatment. If the effect of a number of the groups is simply to cause a slight acid strengthening or acid weakening of the groups in the enzymic site, it can be shown that essentially the same equations for V_S and K_S as given by equations (12) and (13) are obtained. The difference is that the apparent ionization constants are not constant but vary with the pH in regions where the net charge on the enzyme molecule varies with pH. Thus the apparent ionization constants obtained from kinetic data are different from those determined by titration of a simple acid and are more closely related to the apparent ionization constants of particular classes of groups obtained from protein titration data. If the groups which exert acid-strengthening or acid-weakening effects have no effect on the rate of breakdown of the enzyme-substrate complex to yield product, the equation for V_S is

$$V_S = \frac{k_3[\mathrm{E}]_0}{1 + [\mathrm{H}^+]/K_{a0}\exp 2zw + K_{b0}\exp 2zw/[\mathrm{H}^+]} \qquad (27)$$

where K_{a0} and K_{b0} are the values which the apparent ionization constants of the groups in the enzymic site would have in the isoelectric protein. According to equation (27), a plot of V_S versus pH would be symmetrical only if the maximum activity was at the isoelectric point of the protein and the plot of valence versus pH was symmetrical about the isoelectric point. The deviation of the pH variation from that given by equation (12) may be estimated from a knowledge of the isoelectric point and the titration curve of the protein. For accurate calculations, it would be necessary to know the number of ions other than H^+ bound by the protein since this may affect the net charge markedly. The effect of the e^{2zw}

factor is to raise the pK values at pH values above the isoelectric point of the enzyme and to lower them below the isoelectric point and so broadening the plots of V_S and V_S/K_S versus pH. A smaller effect should be obtained at higher salt concentrations where w is smaller.

The second effect concerns the effect of z_E on reactions of the type

$$E^{z_E} + S^{z_S} \rightleftharpoons ES^{(z_E+z_S)}. \tag{28}$$

The difference in free energy between the product and the reactants may be expressed by an equation such as that given earlier for the change in free energy for the dissociation of a proton.

$$\Delta F°/RT = -\ln K_0 + 2z_Sz_Ew \tag{29}$$

where K_0 is the equilibrium constant for the isoelectric protein and w is the same constant encountered in equation (9). Thus, under conditions where the Michaelis constant can be considered to be an equilibrium constant, there will be a dependence of its value on pH even after the ionization of essential groups has been properly taken into account. According to equation (29), only a small effect would be expected in the region around the isoelectric point where the net charge is small. For a negatively charged substrate ion, the dissociation constant for reaction (28) will increase above the isoelectric point of the protein and decrease below the isoelectric point.

The third effect, i.e., the effect of net charge on rate constants, is more difficult to estimate. The activation energy for reaction includes electrical work caused by the charged groups outside the enzymic site, but this electrical work cannot be more than that given by $2z_Sz_EwRT$, for the over-all reaction. If there is no appreciable displacement of charge in the activation process, as for example in the step EF → EM, then no appreciable effect of net electrical charge on the rate of this process is to be expected. However, for an association reaction such as $E + F \rightarrow EF$, the electrostatic effect might be expected to result in a decrease in rate if E and F are of the same electrical charge and an increase in rate if they are oppositely charged.

DISCUSSION

It is really striking that the kinetics of fumarase action depend primarily on the degree of ionization of just two groups. It may be inferred from the bell-shaped nature of the plot of maximum initial velocity versus *p*H that the catalytically active form of the enzyme-substrate complex is that in which one essential group has a proton on it and the other does not. The question as to the significance of this immediately arises. A very attractive explanation is that these two groups per enzymic site are essential because *they are actually involved in the transfer of protons in the enzymic reaction.* This type of mechanism for the fumarase reaction is illustrated in figure 5, which shows only the step in which the enzyme-fumarate complex is converted into the enzyme-L-malate complex. In the hydration of fumarate, a proton is made available at one carbon atom by group R and a hydroxyl group is made available at the other carbon atom by group R′, which accepts a proton from a water molecule. In

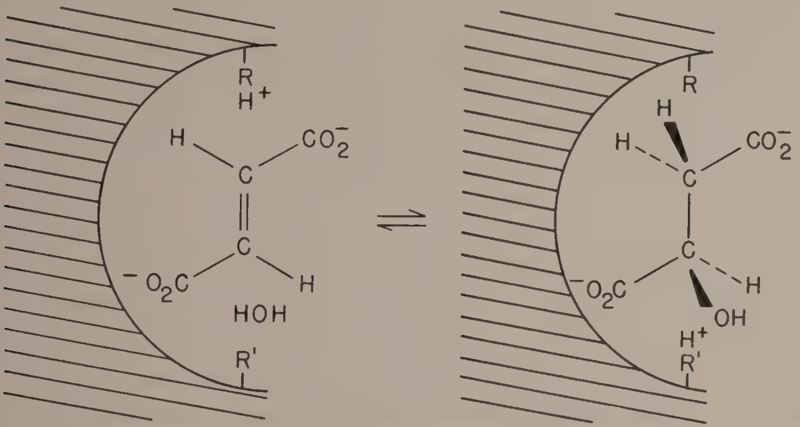


Fig. 5 Mechanism for fumarase action suggested by the study of the effect of *p*H on the kinetics.

the reverse reaction, group R′ donates a proton and group R accepts a proton; the active form of the enzyme-L-malate complex therefore has the proton in the opposite position from the enzyme-fumarate complex. This mechanism offers an explanation for the catalytic inactivity of the complexes containing two protons or no proton. In strongly acidic solutions, the catalytic site spends a large fraction of the time with a proton on the group which functions as a proton acceptor in the catalytic process, and during this time the reaction cannot occur. In strongly alkaline solutions, the catalytic site spends a large fraction of the time with a proton off the group which functions as a proton donor, and during this time the reaction cannot occur. It is of interest that, in fumarase, the pK values of the free enzyme suggest that the two intermediate forms of the enzymic site (HE and EH) exist in nearly equal concentrations in the absence of substrate.

In 1947 Myrbäck gave this kind of interpretation to the fact that the active forms of the urease-urea and saccharase-sucrose complexes are those in which an acid group in the enzymic site is undissociated. He suggested that since the substrate is bound at this point, ‘‘wo so zu sagen örtlich eine hohe H$^+$-Konzentration herrscht,’’ it was reasonable to think that there was acid catalysis of the reaction and that the formation of product occurred only when a proton was available on this acid group.

In 1952, Swain and Brown suggested that some enzymes may be bifunctional catalysts which contain both nucleophylic and electrophylic groups. Barnard and Laidler (’52) discussed this hypothesis in connection with the action of α-chymotrypsin on hydrocinnamic ester and suggested that the active site on the enzyme consists of a carboxyl group and an imidazole group. They pictured a transition state in which the proton of the imidazole group was shared with the ester and a water molecule was shared between the carboxyl group and the carbonyl carbon atom of the ester. Such a transition state is to be distinguished from enzyme-substrate complexes such as those represented by figure 5.

Since bell-shaped plots of maximum velocity versus *p*H are often obtained in hydrolytic reactions, it seems likely that the mechanisms of these enzymic reactions also involve two ionizable groups in the enzymic site. According to this hypothesis, one group donates a proton and the other accepts a proton in the reaction; the location of the proton in the enzyme-reactant complex is therefore just opposite to that in the enzyme-product complex.

The determination of the ionization constants of groups in the enzymic site from kinetic studies is subject to a number of pitfalls. As long as any of the following questions remain unanswered, there will be doubt as to the interpretation of the kinetics in terms of the ionization of essential groups:

1. Have adequate corrections been made for all the irreversible, or slowly reversible, effects of *p*H changes? In other words, have the possibilities of "denaturation" or splitting of a prosthetic group from the enzyme been eliminated as an effect on the rates?

2. Has the effect of the ionization of the substrate, if any, been correctly taken into account? In the simpler substrates, the ionization constants are well known, but in more complicated substrates, this may be a serious problem which has not been dealt with adequately here.

3. Is the enzymic reaction subject to activation or inhibition by components of the buffer, and if so, do these effects change with *p*H over the range investigated? In covering a wide range of *p*H, it is usually necessary to use several buffers, and if these have different specific effects the resulting *p*H dependence of the kinetics might incorrectly be attributed to ionizable groups in the enzyme.

4. Do the kinetic studies extend over a wide enough range of *p*H to provide satisfactory evidence for ionizable groups? There is always the danger that a small bend at the end of

a curve may be taken to indicate an ionization and an ionization constant calculated from insufficient data.

5. Is there activation or inhibition by substrate? In fumarase, the fact that the Haldane equation is obeyed is strong evidence for the absence of such effects in the low concentration region in which the Michaelis-Menton equation is obeyed.

6. Is the magnitude of the expected electrostatic effect on pK values and rates small enough so that it is reasonable that the data can be interpreted in terms of simple mass action expressions? In order to answer this question it is necessary to have some information about the isoelectric point, titration curve, and molecular weight of the enzyme. At pH values far from the isoelectric point, there is the possibility that appreciable changes in kinetic constants with pH may result from electrostatic effects of the rest of the protein molecule.

7. Finally, are there any other complications, such as rapidly reversible unfoldings or dissociation of the protein, which may be reflected as a change in kinetic constants with pH? Proteins being what they are, there are practically infinite possibilities for further complications which would invalidate any simple calculation of ionization constants. However, many of these complications would be apparent from ultracentrifugal, diffusion, viscosity, and other studies of the enzyme over a range of pH.

ACKNOWLEDGMENTS

The author is indebted to Mr. Carl Frieden and Dr. John S. McKinley McKee for the helpful discussions and to Dr. K. L. Laidler for the opportunity of seeing his recent manuscripts prior to publication.

GENERAL DISCUSSION

Ellenbogen[2]: The paper is quite complete in itself but I do have one question. Has Dr. Alberty studied the kinetics in presence of cations at a *p*H where the net charge would be affected?

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[2] Eric Ellenbogen, University of Pittsburgh.

Alberty: You are right, effects should be expected. We have no experimental data, only an experiment near *p*H 7 in which we used lithium, potassium, and ammonium ions and found no difference. I would predict that there would be effects at higher *p*H values.

Kauzmann[3]: In some of Dr. Alberty's equations, the denominator has the form $(1 + K_1[H^+] + K_2/[H^+])$. This denominator gives rise to the bell-shaped activity versus *p*H curves. If, however, the denominator were simply $K_1[H^+] + K_2/[H^+]$ the curves would still be bell-shaped. I wonder if the data require that the number one be included in the denominator. Of course, the theory requires that it be there, but unless both $K_1[H^+]$ and $K_2/[H^+]$ are of the order of magnitude of unity over some range of *p*H values, it is possible to measure only the ratio K_1/K_2 and not the individual values of K_1 and K_2. This seems to be an important limitation on the analysis of data of the type presented in this paper.

Alberty: (Answer added in proof) Since the conference, we have tested the equation for V_S where the denominator is simply of the form $[H^+]/K_a + K_b/[H^+]$. It is readily shown that $K_aK_b = [H^+]_a[H^+]_b = [H^+]_{max}$ where $[H^+]_a$ and $[H^+]_b$ are the hydrogen ion concentrations at half-maximum velocity on the acidic and basic sides and $[H^+]_{max}$ is the hydrogen ion concentration at maximum activity. For any choice of K_a and K_b which satisfies this relationship the same curve width and shape are obtained. The width of such a plot of V_S versus *p*H at half-maximum velocity is 1.2 *p*H units, whereas the widths of the experimental curves in figures 1 and 2 are 1.8 to 2.8 *p*H units.

Koshland[4]: I mentioned the solvent effect in relation to Swain's work to indicate that perhaps under unfavorable solvent conditions the most powerful form of catalysis is used. The ionizing forces might actually be more favorable on the enzyme surface than they are in water, particularly if a metal is involved at the active site. Similarly, an oily or hydrocarbon residue might, in essence, provide a solvent effect which would favor some reactions. Change of solvents in the Menschutkin reaction, for example, makes rate changes in order of magnitude and not just in a few per cent.

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[3] W. Kauzmann, Princeton University.

[4] D. E. Koshland, Brookhaven National Laboratory.

Bell[5]: The question of bound water in peptides and proteins is quite important. The complexity of this binding is illustrated by the fact that β-corticotropin acetate contained about 10% water, which could be removed by heating under high vacuum. However, no water bands were seen in the infrared spectrum of this material.

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[5] P. H. Bell, Stamford Laboratories.

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INFORMAL REMARKS ''BY WAY OF A SUMMARY''

HENRY BORSOOK

To Dr. Hollaender's request that I make a few summarizing remarks about the Symposium, I replied that I do not know enough to make a summary of all that has been said here. Finally, though, I agreed to talk for about 10 minutes by way of a summary — not to give a summary. That I cannot give and I do not think you want to hear it.

In thinking over what I might say, it seemed to me that remarks made to me by two different men about a quarter of a century ago were germane here. The two men were known to many of you — one of them was Edwin Cohn and the other was T. H. Morgan. One became a physical chemist and the other was a geneticist.

Edwin Cohn said to me, ''What I want to do is to do as good physical chemistry on proteins as is being done on sodium chloride.''

I asked Dr. Morgan what he was doing in genetics then and he said, ''I am not doing any genetics. I am bored with genetics. But I am going out to Cal Tech where I hope it will be possible to bring physics and chemistry to bear on biology. I think it will be good for biologists to live in that atmosphere.''

At that time a protein molecule was not a big black ball. It was something very smeary but equally dark. And denaturation was a very mysterious phenomenon. Enzymes were almost as mysterious as they were at the time of the Pasteur-Liebig controversy. We could measure *p*H and a few things like that and that was about all. There was some resentment, I think, among some biologists that their students, the younger people, were even using *p*H meters. In fact, a lot of fun was made about the dominance of *p*H over biology.

Shortly after Dr. Morgan came to Cal Tech (and I came a year after he did), Einstein was visiting our place. He came around to biology, and talking to Dr. Morgan asked him what he was trying to do there, said it was a strange thing for a biologist to be at an Institute of Technology. Dr. Morgan told him what he had told me. Einstein shook his head and said, ''No, this trick won't work. The same trick does not work twice. How on earth are you ever going to explain in terms of chemistry and physics so important a biological phenomenon as first love?''

What I think he had in mind is illustrated by another story in quite a different field. Art historians nowadays, in trying to deemphasize the historical importance of the Renaissance, say that there really were three renascences, and they tell this story: About the time of Charlemagne, about the ninth century, somebody dug up a statue, cleaned it off, and set it up. But they didn't know what it was. They could not make out its meaning. It was a statue of a woman in the nude. She was rather attractive; but why? This was the first renascence. It was a time of finding the facts. Then in the twelfth and thirteenth centuries in the great Scholastic era, they knew very well who this lady was. They knew by then that she was a Venus; and they didn't like it at all. It didn't fit with the prevailing theories, and so they said the statue was a mode of representing the Virgin.

The difference between that renascence and the one that began in the fifteenth century is exemplified by a young man who, looking at this statue, drew the ring from his finger and put it on hers.

What, I think, Einstein was referring to was that we might make out of biology a scholastic science. We have avoided that danger, and this symposium has illustrated that really brilliantly. The physicists and the physical chemists have taken the rings off their fingers and are really married to biology. It is striking that we are not doing any violence to the biological phenomenon or to the biological materials we are trying to describe and understand. The protein molecule is as it is and we now see it in all its curves and folds. The mysterious phenomenon of the denaturation of proteins can be described now in terms of its relation, or lack of relation, to its primary structure and the influence of the secondary and tertiary structures. The enzyme is not mysterious any more. We can now talk about the locus of an enzyme action and mean something clear and definite. We do not have to think of something very special in the way of deformation of the amino acids involved or special prosthetic materials, or special metals. Properties which amino acids have separately, when brought very close together, confer on the locus its enzyme character in a manner that is very much the same as the Swain-Brown model of a catalyst. This is most remarkable.

Even more remarkable, it appears now that the same amino acid side chain that is part of the enzyme locus of fumarase and that subserves the interaction of fumarate and malate, this same side chain — that of histidine, and carrying the same kind of ionic changes — is responsible for the acid-base changes in the combination of oxygen with hemoglobin. Histidine appears to be involved in the site of some proteolytic enzymes. The different workers came to this quite independently and by different methods.

There is a little danger that we may cease to wonder at this really quite wonderful insight that we are now getting. Even the synthesis of protein seemed very mysterious a short time ago — the topic that Dr. Zamecnik and I talked to you about — but is not very mysterious any more.

It seems that we know now where we are going; we know the kind of reactions that are involved. This would have pleased Dr. Morgan, I am quite sure. The geneticists in our company listened to talk about the structure of nucleic acid, and of proteins. Without any difficulty they just take all this information, apply the diagrams, and so on, and out comes a reasonable mechanism for the transmission of hereditary characteristics.

We can feel very happy about our relations with the physicists who have joined us. We are now in a position to make snide remarks about the physicists without being accused of being envious. As you know there are two kinds of physicists.

One is the class of priests, the theoretical physicists, and then there is the ordinary working population, the experimental physicists. The high priests have their own mystic language; from time to time they come out and tell the populace certain things about the meaning of what they have been doing. The two classes are really not very close together.

Among the physicists, the chemists, and the biologists who have been here these last three days there is no such separation. Theory and experiment are very close together. I expect to read in about a year a paper by Dr. Gamow on the isolation of nucleic acid in a better state than any gotten out before, in order to use his information theory to make some sense out of the whole thing. To us this will appear quite natural for Dr. Gamow to do his own biochemistry, even though he came to us as a theoretical physicist.

At the banquet last night, when looking about, you saw that everybody was having a good time. There was that kind of free communication that could happen only if everybody, with all the different names that our jobs are given — like geneticists, biochemists, physical chemists, physicists — talked the same language. Not only did we talk the same language, we all understood, we all believed in the same ideas. We had one world of reference. It is so very exciting, so interesting, that we forgot ourselves. It is very remarkable how the ego just disappeared. I have a feeling that this conference marks the beginning of a new era in biology; we are getting as deep now as we can get. We are talking now about interatomic distances, electron migration, and so on, and still deal with biological phenomena. The physical chemists can find and use the biological phenomena to do interesting physical chemistry and the man who is interested in biological phenomena can use physical chemistry and physics and information theory to do biology. This is the very happy state of affairs. It has come about only in the last couple of years. This is the first conference where so many people from such different disciplines have been brought together and have found so much in common.

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